APPENDIX A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Wolfgang Woloszczuk *et al.*

Serial No.: 10/596,968

Filed: June 30, 2006

For: IDENTIFICATION OF FELINE OR CANINE proBNP

Confirmation No.: 3302

Group Art Unit: 1641

Examiner: FOSTER, Christine E.

Atty. Dkt. No.: SONN:093US

CERTIFICATE OF ELECTRONIC TRANSMISSION

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

May 18, 2010
Date

Travis M. Wohlers

DECLARATION OF ADRAIN BOSWOOD UNDER 37 C.F.R. §1.132

I, Adrian Boswood, the undersigned, declare that:

1. I am a Professor of Veterinary Cardiology at The Royal Veterinary College, University of London. I received a Diploma in the specialty of cardiology from the European College of Veterinary Internal Medicine in 2001. I have research experience in the field of cardiovascular biomarkers. This is confirmed by the list of publications in my *curriculum vitae*. A copy of my *curriculum vitae*, listing my publications in this regard, is attached as Exhibit A.

2. Biomedica paid me £ 500.00 for four hours of time that I spent on this declaration. In 2009, my lab received $10,000 in grant funds from Idexx, and Idexx also provided NT-proBNP test kits to my lab. None of these influenced the factual statements I make or the conclusions I draw in this declaration.

3. I am providing this declaration to discuss the state of knowledge in the field of B-type Natriuretic Peptides (BNPs) as of September 8, 2004. At that time, it was known that multiple forms of BNP exist in the tissues and blood of humans. For example, it was known that human BNP is synthesized as a preprohormone, known as preproBNP, in myocardial cells. The preproBNP is then cleaved to form a signal peptide and proBNP. The proBNP is further processed into an N-terminal fragment (NTproBNP) and a C-terminal fragment (BNP-32), which are detectable in blood. In humans, it was known that BNP exists primarily as proBNP, rather than BNP-32, in human blood. *See* Exhibit B (European Patent Application Number 1016867A1). In other species, namely cats and dogs, less was known about BNPs in general and proBNP specifically.

4. Although some information regarding the forms of human BNP present in tissues and in blood was known, much remained unknown about human BNP molecules. For example, studies indicated that there were additional and yet unidentified BNP forms circulating in human plasma. Some proBNP molecules exhibited a much higher molecular weight than would be expected for a single proBNP molecule, indicating that BNPs may bind to other molecules or to each other. For this reason, the authors of a 2004 review article concluded:

> Thus, although proBNP and its N-terminal fragments seem to associate to something in cardiac tissue and plasma, the underlying mechanism still needs to be determined. It is nevertheless important to emphasize that such oligomerization may have a major influence on antibody detection and assay performance.

Exhibit C, p. 1507, col. 1 (Goetze, Biochemistry of Pro-B-Type Natriuretic Peptide-Derived Peptides: The Endocrine Heart Revisited, *Clin. Chem.* **50:**1503–10, 2004) (emphasis added).

5. Those statements indicate that, as of 2004, it was unknown how many forms of BNP molecules are circulating in human plasma. Moreover, because "proBNP and its N-terminal fragments seem to associate to something in cardiac tissue and plasma," it was not possible to predict that an antibody against a particular amino acid region of proBNP could detect proBNP or a particular N-terminal fragment of proBNP.

6. Further emphasizing the general uncertainty surrounding BNP structure in 2004, the Goetze review article explains:

> [O]ur present understanding of the structural biochemistry is still far from complete. In particular, cellular synthesis, including posttranslational maturation and metabolism of the peptides, is poorly characterized. Further elucidation of the molecular heterogeneity could provide important biological insight into the endocrine heart and could likely have important diagnostic consequences. Above all, the different proBNP-derived peptides may not always be equal markers of the same pathophysiologic processes. In addition, differences in elimination may introduce new boundaries for diagnostic use.

Exhibit C, p. 1508, col. 1 (emphasis added).

7. In summary, the Goetze article indicates that, as of 2004, much remained unknown about BNP. For example, due to the molecular heterogeneity of BNP, it was unclear how many forms of BNP existed in tissues and in blood. Moreover, it was unclear which amino acid regions of the various forms of BNP could be detected using antibodies. Finally, it was unclear which of the various BNP forms would be useful as markers of pathophysiologic

processes, such as a pathophysiologic process that accompanies or precedes a cardiac disease.

8. Although it was reported that human BNP exists primarily in human blood as proBNP, Exhibit B, col. 2, lines 45–53, it remained unknown, in September 2004, how many forms of BNP are present in other species, such as cats and dogs, and which of those BNP forms would predominate in the plasma in those species. In fact, growing evidence indicated that the forms of BNPs present in blood and tissues, as well as the functions of BNPs, varied across species.

9. For example, one study published in 2002 explained:

> [P]reproBNP of eight mammalian species showed variations in length and sequence structures. This supports the species-specific actions of BNP across species.

Exhibit D, p. 188, col. 2 (Liu *et al.*, Cloning and Characterization of Feline Brain Natriuretic Peptide, *Gene* **292:**183–90, 2002). Comparing BNP sequences across species, the authors grouped the species as follows: (a) rat and mouse BNPs are closely related; (b) cattle, sheep, and swine BNPs are closely related; (c) cat and dog BNPs are closely related; and (d) human BNP is in a "distinct group as compared to the other species." Exhibit D, p. 187, col. 2. Regarding human BNP, the authors specifically noted that "human preprop[e]ptide has many unique sequences and appears to have evolved independently from other species." Exhibit D, p. 188, col. 1.

10. Another study also noted the structural variation of BNPs across species:

> BNP differs across species, with only short segments retaining sequence homology. In addition, there are species-specific variations in the structure of the non-guanylyl cyclase-linked natriuretic peptide C (NP_C)

> receptor or clearance receptor, which is likely to affect the metabolism of BNP.

Exhibit E, p. 369, col. 1(Thomas *et al.*, Haemodynamic Action of B-Type Natriuretic Peptide Substantially Outlasts its Plasma Half Life in Conscious Dogs, *Clin. Exp. Pharmacol. Physiol.* **30:**369–75, 2003). In particular, that study indicated the likelihood that BNP is metabolized differently across species.

11. Such structural heterogeneity across species could have at least two consequences. First, BNP could have different fragmentation patterns in different species. Second, BNP forms could have different half-lives in different species. Indeed, studies confirmed that BNP does have different fragmentation patterns across species, and the half-life of BNP molecules does vary across species.

12. First, studies confirmed that one species may have different forms of BNP as compared to a different species. For instance, one 32-amino-acid form of mature BNP was known in human, dog, and pig; one 45-amino-acid form of mature BNP was known in mouse and rat; two forms of mature BNP were known in sheep (one form having 26 amino acids and one form having 29 amino acids); two forms of mature BNP were predicted in cow (one form having 26 amino acids and one form having 29 amino acids); and three forms of mature BNP were predicted in cat (one form having 26 amino acids, one form having 29 amino acids, and one form having 35 amino acids). Exhibit D, p. 188, col. 1. Thus, it was known that one species may have different forms of circulating BNPs as compared to a different species.

13. Second, studies confirmed that the half-life of BNP molecules varies across species. For example, studies showed that the half-life of canine BNP is far shorter than the half-life of BNP in rats, sheep, or humans. Exhibit E, p. 369, col. 2. Thus, it was known that the stability of BNP molecules varies across species.

14. Taken together, the Liu *et al.* and MacDonald *et al.* studies demonstrate that, as of September 8, 2004, it was unknown how many forms of circulating BNP were present and/or could be detected in non-human animals, such as cats or dogs. In particular, because it was known that the structures and half-lives of BNP forms vary across species, it was not possible to predict that a circulating form of BNP that is present and can be detected using antibodies in humans would also be present and able to be detected using antibodies in cats or dogs.

15. Finally, the structural variation of BNPs across species "supports the species-specific actions of BNP across species." Exhibit D, p. 188, col. 2. Such species-specific functions for BNP indicate that, based on the discovery of a particular scientific phenomenon regarding BNP in one species (*e.g.*, human), one could not reasonably predict that the same phenomenon will exist in a different species (*e.g.*, dog or cat).

16. Indeed, such species-specific functions of BNP were well-documented as of September 8, 2004. For example, as noted above, the half-life of canine BNP is far shorter than the half-life of BNP in rats, sheep, or humans. However, although canine BNP had a half-life of only approximately 90 seconds, canine BNP's effects on blood pressure and blood flow endured for more than 20 minutes after the infusion of BNP. Exhibit E, p. 369, col. 1. The surprising fact that the effects of canine BNP last substantially longer than

canine BNP is present in the blood signifies that BNP signaling in dogs is distinct from BNP signaling in other species, such as rat, sheep, or human.

17. Further highlighting the functional variation of BNPs across species, plasma BNP increases with age in humans, but "there is no correlation between plasma [BNP] and age in dogs." Exhibit F, p. 175, col. 1 (MacDonald *et al.*, Brain Natriuretic Peptide Concentration in Dogs with Heart Disease and Congestive Heart Failure, *J. Vet. Intern. Med.* **17**:172–77, 2003). That study demonstrates that not all observations regarding circulating concentrations of human BNP can be extrapolated to canine BNP.

18. Finally, a 2001 report provided further evidence of the species-specific actions of BNP:

> The disparity between the current early onset of LV ANP and BNP gene expression in the rabbit model and the late onset in the dog model is remarkable and may indicate that activation of cardiac natriuretic peptide expression in heart failure differs between species.

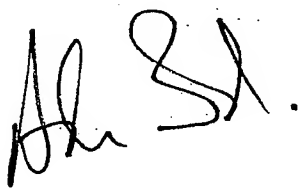
Exhibit G, p. 606, col. 2 (Luchner *et al.*, Differential Expression of Cardiac ANP and BNP in a Rabbit Model of Progressive Left Ventricular Dysfunction, *Cardiovasc. Res.* 51:601–07, 2001). Again, such studies support the notion that a discovery regarding BNP in one species does not predictably translate to BNP in another species. In fact, this study indicates that while expression of BNP and similar molecules may provide a useful indicator of heart failure in one species, expression of such molecules may not provide a useful indicator of heart failure in a different species.

19. Taken together, the studies discussed herein demonstrate that, as of September 8, 2004, it was known that BNP structure and function differed across species, such that findings regarding BNP in one species would not predictably translate to a different species.

More specifically, the following were neither known nor could be predicted: (1) how many forms of BNP were present in the tissues and blood of humans and other species; (2) which forms of circulating BNP would be predominant in species such as cats and dogs; (3) whether any forms of circulating BNP would be stable enough to be detected by antibodies in cats or dogs; and (4) which forms of BNP would be useful as markers of pathophysiologic processes.

20. For the foregoing reasons, as of September 8, 2004, it was unknown whether circulating proBNP could be detected in dogs or cats. Furthermore, it was not possible to predict that antibodies directed against a particular amino acid region of dog proBNP could detect circulating proBNP in dogs. Likewise, it was not possible to predict that antibodies directed against a particular amino acid region of cat proBNP could detect circulating proBNP in cats.

21. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code.



__Tuesday, May 18, 2010_____ __Adrian Boswood_____
Date

EXHIBIT A

Curriculum vitae of Professor Adrian Boswood

Name	Professor Adrian Boswood	
Address	The Royal Veterinary College University of London Hawkshead Lane North Mymms Hatfield Hertfordshire AL9 7TA United Kingdom	
Qualifications	1986	Bachelor of Arts (BA) University of Cambridge
	1989	Bachelor of Veterinary Medicine (VetMB) University of Cambridge
	1990	Master of Arts (MA) University of Cambridge
	1996	Diploma of Veterinary Cardiology (DVC) Royal College of Veterinary Surgeons
	2001	Diploma of the European College of Veterinary Internal Medicine (Companion Animals) in the sub specialty of cardiology (DipECVIM-CA (Cardiology))
Employment	July 1989-October 1990	Veterinary Surgeon Mixed, predominantly small animal practice.
	October 1990	Joined the Royal Veterinary College where all subsequent positions have been held
	October 1990-September 1991	Intern in Small Animal Medicine and Surgery
	September 1991-November 1993	Resident in Small Animal Internal Medicine
	December 1993-October 1996	Clinical Instructor in Internal Medicine
	November 1996-2001	Clinical Lecturer in Internal Medicine
	2001-2009	Senior Lecturer in Internal Medicine/Cardiology
	2009-to date	Professor of Veterinary Cardiology
Professional and society memberships		European College of Veterinary Internal Medicine
		British Small Animal Veterinary Association
		Veterinary Cardiovascular Society
		Royal College of Veterinary Surgeons

Publications (reverse chronological order)

Those that indicate an expertise in biomarkers are italicized

Original articles

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EXHIBIT B

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 016 867 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
05.07.2000 Bulletin 2000/27

(21) Application number: **98941797.7**

(22) Date of filing: **10.09.1998**

(51) Int. Cl.[7]: **G01N 33/53**

(86) International application number:
PCT/JP98/04063

(87) International publication number:
WO 99/13331 (18.03.1999 Gazette 1999/11)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **11.09.1997 JP 24668497**

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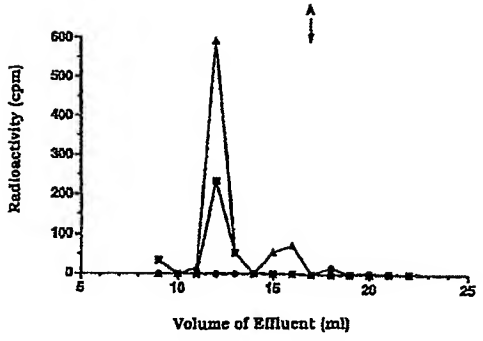
(54) IMMUNOASSAY METHOD FOR BNP

(57) An immunoassay specific for mammalian γ-BNP derivatives, which uses the first antibody reactive with mammalian α-BNP and the second antibody reactive with mammalian prepro-BNP or γ-BNP derivatives and not α-BNP, and at least one of the first and the second antibodies is optionally labeled detectably labeled or immobilized.

Fig. 3



EP 1 016 867 A1

Description

TECHNYCAL FIELD

[0001] The present invention relates to an immunoassay for the brain natriuretic peptide (BNP) which is a member of natriuretic peptide family, more specifically, it relates to an immunoassay for γ -BNP and derivatives thereof.

BACKGROUND ART

[0002] Natriuretic peptide family includes three members, i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and type C natriuretic peptide (CNP). Among them, ANP and BNP are cardiac hormones which are mainly biosynthesized in and secreted from the heart. ANP and BNP are similar in structure. ANP is a peptide of 28 amino acids with a ring (circular) structure formed by a disulfide bond between the 7th and the 23rd cysteine residues, while BNP is a peptide of 32 amino acids with a ring structure formed by a disulfide bond between the 10th and the 26th cysteine residues. These mature peptides of 28 and 32 amino acids have been considered to be produced from respective precursor when a leader sequence is cleaved off intracellularly or at the time of secretion. That is, there has been reported that human BNP is first synthesized as a preprohormone (hereinafter, referred to as prepro-BNP) in myocardial cells, which is split before or at the time of secretion between Ser^{26}-His^{27} to give pro-BNP (hereinafter, referred to as γ-BNP), and which is further split between Arg^{102}-Ser^{103} to give BNP-32 (hereinafter, referred to as α-BNP) and BNP(1-76), and that the former exhibits the activity. It has been considered that at least the ring structure must be remained for the expression of activity.

[0003] The secretion of cardiac hormones being stimulated by various heart diseases, it well reflects the change in the cardiac functions. The secretion of ANP is accelerated mainly when the atrium undergoes a load, while the biosynthesis and secretion of BNP are stimulated when the ventricle undergoes a load. Accordingly, both ANP and BNP are useful as indicators in the diagnosis of heart disease. As the progress of investigation in the *in vivo* role of respective hormone, the advantageous features of BNP as an indicator for diagnosing heart disease have become clear. For example, the blood concentration of BNP is only 1/6 of ANP in a normal subject but it becomes higher than ANP in patients of heart failure or the like; the blood concentration of BNP increases in the case of heart failure like ANP, and the plasma concentration of BNP often exceeds that of ANP reflecting more accurately the severity of heart dysfunction; the plasma concentration of both ANP and BNP elevates in peripheral blood and elevation rate is higher in BNP. Moreover, BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For these reasons, the usefulness of BNP measurement has been suggested (Y. Saito et al., Mebio, 12(5), 28, 1995).

[0004] Under the conditions, an immunoassay which utilizes antiBNP antibody and is applicable to the diagnosis of cardiac insufficiency has been proposed. Japanese Patent Publication (KOHYO) 7-507210 describes a method of measuring γ-BNP (1-76) produced by biodegradation by protease or the like. However, this method is directed to γ-BNP (1-76) which lacks the portion(s) essential for the expression of activity such as ring structure and, therefore, cannot determine the hormone activity directly.

[0005] An assay kit for the measurement of α-BNP having natriuretic activity has been marketed ("BNP-32", Peninsula). With this kit, degradation products of α-BNP in blood including fragments lacking activity due to the deletion of C-terminal region can also been measured. Taking the low blood concentration of BNP into consideration, the measurements involving the degradation products cannot be disregard. Accordingly, the said method connotes disadvantages to be an assay for BNP in the establishment of an accurate diagnosis of heart failure.

[0006] As a kit for the measurement of BNP free from the disadvantages above has been marketed ("SHIONORIA BNP", Shionogi), which characteristically uses an antibody recognizing the structure essential for the expression of activity. However, this method would be affected significantly by the process for collecting and storing blood sample, because α-BNP is extremely instable in collected blood. It is, therefore, suggested that the sample should be specifically treated by, for instance, adding an agent for inhibiting degradation into a blood collecting tube or maintaining the sample at low temperature so as to obtain reliable data. Such procedures may hamper the extensive clinical application of the said BNP assay kit.

DISCLOSURE OF INVENTION

[0007] The present inventors have conducted research intensively for the purpose of establishing an accurate method of diagnosing cardiac diseases involving BNP and found that BNP exists in blood in the form of γ-BNP or its degradation product which at least comprises structurally the α-BNP moiety (hereinafter, they are referred to as "γ-BNP derivative"), and not in the form of α-BNP which has so far been considered to be dominant. The inventors have also found that γ-BNP is more stable than α-BNP in blood, that is, one role of the N-terminal structure of γ-BNP, among many, would be the stabilization of BNP. The above indicates that an organism biosynthesizes at least 2 kinds of BNP molecule which share the BNP activity but differ in half-life.

These findings led the present inventors to have a view that it is indispensable to establish a method specific for not only α-BNP but also γ-BNP to accomplish an accurate diagnosis of cardiac diseases.

[0008] The present invention provides an immunoassay specific for mammalian γ-BNP derivatives, characterized in that it uses the first antibody which is reactive with mammalian α-BNP and the second antibody which is reactive with mammalian prepro-BNP or γ-BNP derivatives and not reactive with α-BNP.

[0009] As used herein, the term "mammalian α-BNP" refers to a peptide of low molecular weight having natriuretic activity which is derived from mammalian prepro-BNP or γ-BNP through the removal of N-terminal region as a result of processing at the carboxy terminus of processing signal sequence. In case of human BNP, α -BNP is a peptide consisting of C-terminal 32 amino acids (Nos. 103-134) of the amino acid sequence of SEQ ID NO: 1 and having a ring structure. The carboxy terminus of processing signal sequence on the prepro-BNP molecule varies slightly depending on the species. For example, it is No. 102 Arg in case of human BNP while it is No. 100 amino acid in case of porcine or canine BNP.

[0010] As used herein, the term "mammalian γ-BNP" refers to a pro-BNP comprising a partial peptide of 32 amino acids corresponding to α-BNP at the carboxy terminal region. In case of human γ-BNP, it is pro-BNP of 108 amino acids from No. 27 His to No. 134 His of the amino acid sequence of SEQ ID NO: 1. The term "prepro-BNP" refers to a peptide of 134 amino acids from No. 1 Met to No. 134 His of the amino acid sequence of SEQ ID NO: 1 in case of human.

[0011] As used herein, the term "mammalian γ-BNP derivative" refers to a peptide fragment derived from mammalian prepro-BNP or γ-BNP through mainly the *in vivo* protease reaction, which fragment includes or is larger than α-BNP. Although γ-BNP derivative would comprise a molecule of the same or smaller size compared to γ-BNP in general, it may comprise a molecule larger than γ-BNP. Otherwise specifically mentioned, as used herein, the term "γ-BNP derivative" includes γ-BNP itself.

[0012] The term "stable", when used herein in connection with BNP, means that a BNP molecule maintains the C-terminal ring structure including C-terminus of BNP and the natriuretic activity after undergoing the degradation by protease, and that the said activity is not significantly decreased even 24 hours from the collection of blood samples. In light of this definition, the γ-BNP derivative as the target substance (analyte) of the present immunoassay is stable.

[0013] On the other hand, the term "unstable" means that a BNP sample undergoes degeneration by protease at the C-terminal region and that the natriuretic activity is significantly decreases 24 hours from the collection of blood samples. In light of this definition, α-BNP is unstable.

BRIEF DESCRIPTION OF DRAWINGS

[0014]

Fig. 1 is a chromatogram obtained in an α-BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample. In Fig. 1, A indicates the position of elution of α-BNP.

Fig.2 is a chromatogram obtained in an α-BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample different from that shown in Fig. 1. In Fig. 2, A indicates the position of elution of α-BNP.

Fig. 3 is a chromatogram obtained in an immunoassay specific for γ-BNP wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample same as those shown in Fig. 2. In Fig. 3, A indicates the position of elution of α-BNP.

Fig.4 is a graph showing the relationships between the storing time and BNP immunoreactivity of γ-BNP kept in human plasma at 25°C.

Fig.5 is a graph showing the relationships between the storing time and BNP immunoreactivity of α-BNP kept in human plasma at 4°C.

BEST MODE FOR CARRING OUT THE PRESENT INVENTION

[0015] In one embodiment of the present invention, it is related to a method which uses two antibodies, wherein the first antibody is reactive with mammalian α-BNP and the second antibody is reactive with prepro-BNP or γ-BNP derivatives and is not reactive with α-BNP.

[0016] Antibodies used in the present method can be monoclonal or polyclonal antibodies. The first antibody can be prepared according to a method known in the art using as an antigen human α-BNP which is commercially available or chemically synthesized, or a partial peptide thereof. Alternatively, a monoclonal antibody appended to a commercially available α-BNP assay system (kit) for measuring α-BNP ("SHIONORIA", Shionogi) is also available, which is reactive with the C-terminal region of α-BNP.

[0017] As the second antibody, any antibody can be used subject that it meets the conditions above. Preferred examples of such antibody include those specific for the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1 or metabolites thereof. The γ-BNP derivatives as an analyte to be measured by the present method preferably include at least the partial amino acid sequence shown by the amino acid Nos. 27-134 of SEQ ID NO: 1, in case of human BNP. Accordingly, in a preferred embodiment of the present invention, a special attention is preferably paid in the selection of an antigen to obtain an antibody capable of recognizing the amino acid sequence shown by amino acid Nos. 27-102. The preparation of such an antibody

can be carried out by any one of methods known in the art. Theoretically, γ-BNP molecule can be cleaved by protease at sites corresponding to No. 47 (Arg), No. 53 (Lys) and No. 72 (Arg) in the amino acid sequence of SEQ ID NO: 1, and, therefore, an antibody recognizing an amino acid sequence shown by amino acid Nos. 73-102 of SEQ ID NO: 1 can be used as the second antibody.

[0018] The assay of the present invention can be either a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

[0019] At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support.

[0020] The method for labeling or immobilizing an antibody is known to one ordinary skilled in the art. Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, and particles. The labeling of an antibody can be carried out according to a method known to one ordinary skilled in the art, for example, that described by Kono et al. (Kaku-Igaku Gijutu, 13(1), 2, (1993)).

[0021] The present invention further provides a kit for immunoassay specific to mammalian γ-BNP derivatives, characterized in that it comprises two antibodies wherein the first antibody is reactive with mammalian α-BNP and the second antibody is reactive with mammalian prepro-BNP or γ-BNP derivatives and is not reactive with α-BNP.

[0022] The kit of the present invention can be for a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

[0023] At least one of the first and the second antibodies may be labeled detectably or immobilized on a solid support. The kit of the present invention may further contain a means for detecting the label. Examples of label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, or particles.

[0024] The following examples and test examples are provided to further illustrate the present invention, without limiting the scope thereof.

Example 1

Measurement of γ-BNP Derivatives by Sandwich IRMA

[0025] Throughout the following Examples, the ordinary reagents used are of special grade supplied by Wako Pure Chemicals Industries, Ltd. or Nacalai Tesque, Inc. The bovine serum albumin (BSA) was purchased from Sigma.

(1) Preparation of Plasma Sample

[0026]

1) Venous blood was collected from patients of cardiac disease or healthy volunteers and placed in blood-collecting tubes containing EDTA and aprotinin (500 KIU/l, Sigma) derived from bovine lung. The tubes were centrifuged (x2000 g at 4°C) for 5 minutes with H-107RGA (Kokusan) to separate blood cells. The resultant plasma samples were freezed and stored at -80°C until use.

2) The plasma samples prepared in 1) above from patients of cardiac disease or healthy volunteers were fractionated by gel filtration HPLC system LC10A (Shimadzu) equipped with Superdex 75 10/30 column (Pharmacia). After equilibrating the column with 0.1 M phosphate buffer (pH 7.5, 0.3M NaCl, 5 mM EDTA) at flow rate of 1 ml/min, 1 ml of plasma sample was injected and 1 ml each of effluent eluted from the column was collected. Each fraction was subjected to the measurement by assay systems for measuring α-BNP or γ-BNP as described in (2)-2) and (2)-3) below, respectively.

(2) Construction of Assay System For Measuring α-BNP- or γ-BNP Derivative

1) In the assay system, the following peptides, antibodies and kits were used.

[0027]

- Human α-BNP (Peptide Institute)
- Antibody against the amino terminal region of γ-hBNP (amino acid Nos. 27-64 of SEQ ID NO: 1) (Peptide Institute)
- Monoclonal antibody against the carboxy terminal structure of α-BNP (BC203). BC203 is an immobilized antibody appended to SIONORIA BNP kit (Shionogi), wherein a monoclonal antibody directed to the carboxy terminal structure of α-BNP is immobilized on beads.
- Monoclonal antibody against the ring structure of α-BNP (KYBNPII). KYBNPII is a monoclonal antibody appended to the SIONORIA BNP kit (Shionogi), which is directed to the ring structure (112-128) of α-BNP, and is labeled with ^{125}I.

2) Measurement of Plasma Fraction by Assay System for α-BNP

[0028] The measurement of α-BNP was carried out by commercially available "SHIONORIA BNP kit" (Shionogi). The assay is based on sandwich IRMA (Immunoradiometric Assay) which uses a monoclonal antibody KYBNPII specific for the ring-structure of α-BNP and another monoclonal antibody BC203 specific for the carboxy terminal structure of α-BNP. The assay

was carried out in accordance with the supplier's instructions.

[0029] That is, 100 μl each of samples to be assayed or standard solutions (0, 4, 10, 150, 600 or 2000 pg/ml of α-BNP solution) were dispensed into a polystyrene test tube. To the test tube was added 200 μl of iodine-labeled anti-BNP antibody (^{125}I) solution, followed by one polystyrene bead on which anti-BC203 antibody has been immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4 °C. After washing twice with 2 ml of washing solution, radioactivity was measured on γ-counter ARC-600 (Aloka) The results are shown in Figs. 1 and 2.

3) Measurement of Plasma Fraction by Assay System For γ-BNP Derivative

[0030] An antibody against amino terminal portion (Nos. 27-64) of γ-hBNP was first labeled with ^{125}I.

[0031] IgG was purified from anti-serum (Peptide Institute) raised against amino terminal portion (amino acid Nos. 27-64 of SEQ ID NO: 1) of γ-hBNP using MASPII kit (Bio-Rad) and displaced with 0.5 M phosphate buffer (pH 7.5) using Centricon 30 (Amicon). The labeling of antibody was carried out by the chloramine T method. To a glass tube was dispensed 170 μl of purified IgG solution (77.6 μg, IgG), and 10 μl of Na^{125}I solution (34.2 MBq, Amersham) was added. After addition of 0.1 % chloramine T solution (20 μl), the mixture was vigorously stirred at room temperature for 30 seconds. The reaction was quenched by adding 20 μl of 0.25 % sodium pyrosulfite solution and 20 μl of 5% aqueous potassium iodide solution. When the reaction mixture was treated with Ampure SA column (Amersham) to remove unreacted ^{125}I and to desalt, solution containing ^{125}I-labeled antibody was obtained.

[0032] The sandwich IRMA was then carried out in plasma fractions by using the resultant antibody and polystyrene beads on which an antibody recognizing the carboxy terminal structure of α-BNP (BC203).

[0033] To a polystyrene tube was placed 100 μl each of samples to be assayed, followed by 200 μl of 0.1 M phosphate buffer (pH 7.5, 0.3M, 5 mM EDTA, 0.2% BSA and 500 KIU/l bovine lung aprotinin (Sigma)) and one polystyrene bead on which BC203 antibody has been immobilized. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4 °C. After washing twice with 2 ml of washing solution, 300 μl of ^{125}I-labeled antibody solution was added. The mixture was stirred and allowed to react by leaving stand for 18 hours at 4 °C. After washing twice with 2 ml of washing solution, radioactivity was measured on γ-counter ARC-600 (Aloka) The results are shown in Fig. 3.

(3) Results

[0034] Figs. 1, 2 and 3 show the chromatograms of gel filtration HPLC of plasma samples obtained from patients, wherein A is the position of elution of α-BNP.

[0035] Fig. 1 shows the result of the measurement conducted by the α-BNP assay kit described in (2)-2) above. In the Fig. 1, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of effluent eluted from the column as measured by SHINORIA BNP kit. The solid triangle, open square, and open rhombus respectively represent the measurements in different plasma samples.

[0036] Fig.2 shows the result of the measurement conducted by the α-BNP assay kit described in (2)-2) above in samples different from those shown in Fig. 1. In the Fig. 2, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of effluent eluted from the column as measured by SHINORIA BNP kit. The solid triangle and solid square respectively represent the measurements in different plasma samples.

[0037] From Figs. 1 and 2, it is revealed that there exist substances of molecular weight larger than α-BNP and having BNP-like immunoreactivity in the plasma of patients of cardiac disease, and that they are the major substances having BNP immunoreactivity.

[0038] Fig.3 shows the results of the measurement conducted by γ -BNP assay kit described in (2)-3) above in the same samples those shown in Fig. 2. In Fig. 3, the vertical axis represents the radioactivity measured by the γ-BNP immunoassay system and the horizontal axis the volume of effluent eluted from the column. The solid circle represents the measurements of α-BNP, which obtained after fractionating human α-BNP solution by HPLC in a similar manner as described in the case of plasma.

[0039] From Fig. 3, it is revealed that the immunoassay specific for γ-BNP derivative of the present invention can detect the major substances with BNP immunoreactivity, but cannot α-BNP at all.

[0040] The results above indicate that the immunoassay for γ-BNP of the present invention is insensitive to α-BNP but specific to γ-BNP derivatives. Further, it has also been revealed that γ-BNP is the major substance having BNP immunoreactivity.

Test Example 1

Stability of γ-BNP Derivatives and α-BNP in Plasma

[0041] Fractions suspected to contain γ-BNP derivative were collected from those obtained by treating plasma samples collected from patients of cardiac disease by gel filtration HPLC. Venous blood was collected from healthy volunteers using blood-collecting tubes containing EDTA in the absence of bovine lung aprotinin. Plasma samples (the minimum detection limit of α-BNP < 4 pg/ml) were prepared in a manner similar to that described in (1)-1) above. The plasma sample was allowed to stand for 0, 2, 6, 24 hours at room tempera-

ture (25°C) after addition of the fraction. The stability of BNP derivative was evaluated by determining the BNP immunoreactivity in the plasma sample by means of SHIONORIA BNP kit for assaying α-BNP.

[0042] Separately, the stability of α-BNP was evaluated using a plasma sample prepared by adding chemically synthesized α-BNP to plasma collected from healthy volunteers and standing for 0, 2, 6 and 24 hours at 4°C in the absence of bovine lung aprotinin as described above. The BNP immunoreactivity in the plasma sample was determined by SHIONORIA BNP kit in the same manner as above.

[0043] The stability of γ-BNP derivatives and α-BNP in plasma samples are shown in Figs. 4 and 5, respectively.

[0044] From Fig. 4, it is revealed that γ-BNP derivatives do not lose significantly the immunoreactivity compared with the initial activity even after 24-hour-standing at 25°C. From Fig. 5, by contrast, it is revealed that α-BNP loses the immunoreactivity to about 40% based on the initial activity after 24-hour-standing at 4°C.

[0045] The above results demonstrate that α-BNP is far less stable compared with γ-BNP derivative in blood and that the latter is much more suited in the diagnosis of cardiac diseases than the former.

INDUSTRIAL APPLICABILITY

[0046] As mentioned above, the BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For this reason, the usefulness of BNP measurement has been proposed.

[0047] The immunoassay of the present invention allows to determine specifically γ-BNP derivatives without measuring α-BNP. Accordingly, the present immunoassay can be a clinically significant means for diagnosis and prognostic monitoring of heart failure, which leads to conclusion/judgment somehow different from those resulted from conventional BNP assay.

[0048] Further, it is herein disclosed for the first time that γ-BNP, which is a target substance to be assayed by the present method, is stable in blood. Therefore, immunoassay of the present invention provides stable and reliable clinical data without being affected by the process of collecting or storing samples, or the time from the collection until measurement. Further, the immunoassay of the present invention does not require any special pretreatments of blood sample and therefore gives clinical data conveniently, thereby contributing to the establishment of highly accurate diagnosis of cardiac diseases.

Claims

1. An immunoassay specific for mammalian γ-BNP derivatives, characterized in that it uses the first antibody reactive with mammalian α-BNP and the second antibody reactive with mammalian prepro-BNP or γ-BNP derivatives and not α-BNP.

2. The immunoassay of claim 1, wherein the γ-BNP derivatives comprise the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1.

3. The immunoassay of claim 1, wherein the second antibody is specific for the amino acid sequence shown by the amino acid Nos. 27-102 of SEQ ID NO: 1.

4. The immunoassay of any one of claims 1 to 3, wherein at least one of the first and the second antibodies is detectably labeled or immobilized.

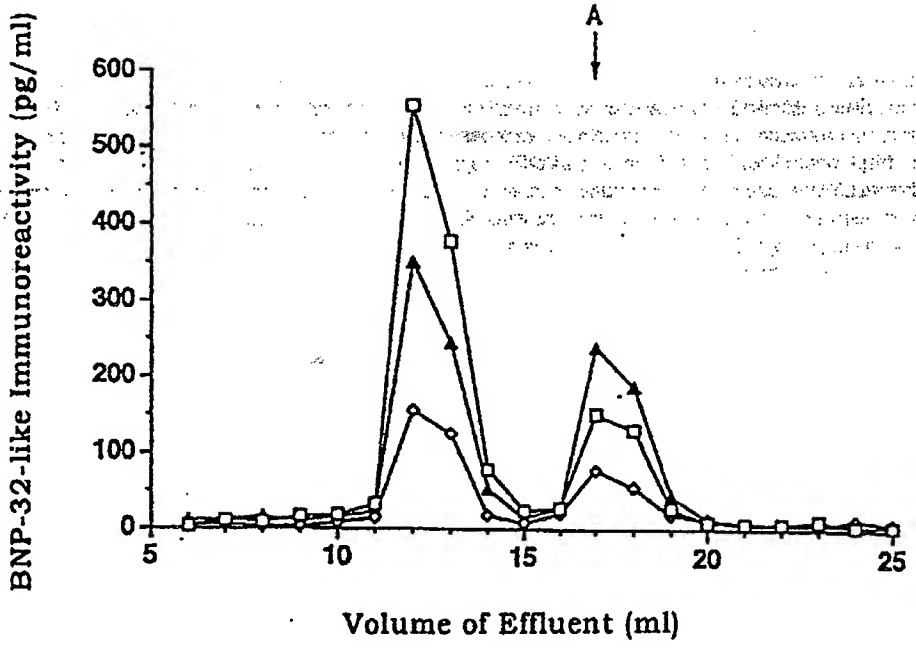
5. The immunoassay of any one of claims 1 to 3, wherein the detectable label is a radioactive isotope, an enzyme, a fluorescent substance, a luminescent substance, or a particle.

6. A kit for immunoassay specific for mammalian γ-BNP derivatives, characterized in that it comprises the first antibody reactive with mammalian α-BNP and the second antibody reactive with mammalian prepro-BNP or γ-BNP derivatives and not α-BNP.

7. The kit of claim 6, wherein at least one of the first and the second antibodies is detectably labeled or immobilized.

8. The kit of claim 7 which further comprises a means for detecting the label.

Fig. 1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600
500
400
300
200
100
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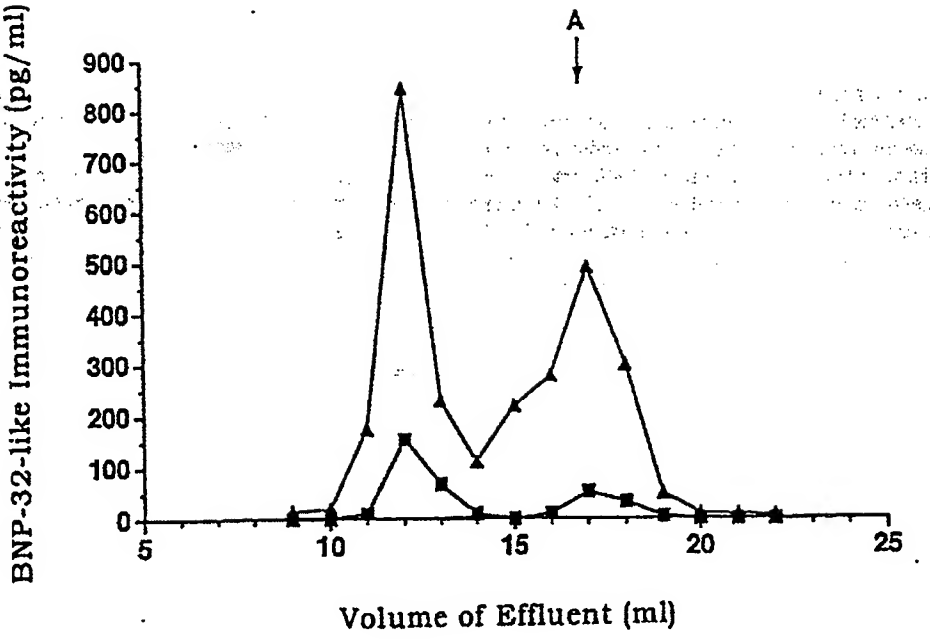
5
10
15
20
25
BNP-32-like Immunoreactivity (pg/ml)
Volume of Effluent (ml)

Fig. 2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400
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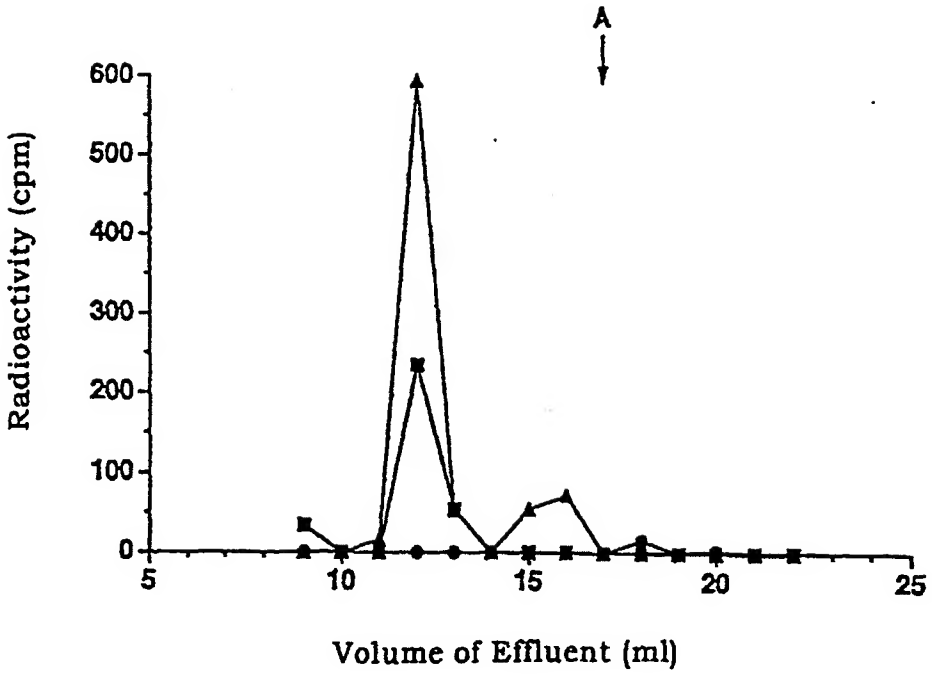
20
25
BNP-32-like Immunoreactivity (pg/ml)
Volume of Effluent (ml)

Fig. 3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200
100
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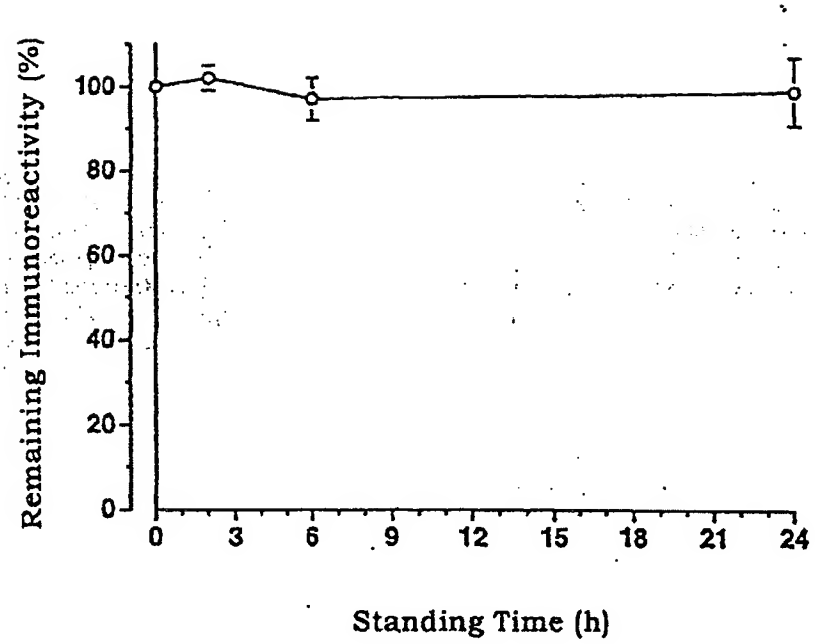
Radioactivity (cpm)
5
10
15
20
25
Volume of Effluent (ml)

Fig. 4


Remaining Immunoreactivity (%)
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80
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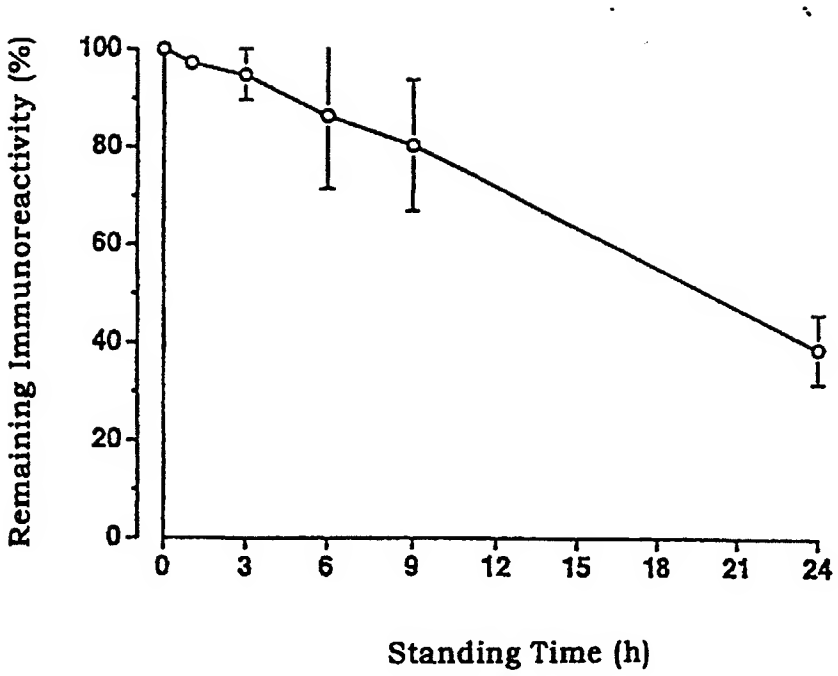
40
20
0
0
3
6
9
12
15
18
21
24
Standing Time (h)

Fig. 5


Remaining Immunoreactivity (%)
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
Standing Time (h)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/04063

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1926-1998 Toroku Jitsuyo Shinan Koho 1994-1998
Kokai Jitsuyo Shinan Koho 1971-1995

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, REGISTRY

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 7-507210, A (Medinnova SF), 10 August, 1995 (10. 08. 95) & WO, 93/24531, A	1-8
Y	JP, 3-297392, A (Shionogi & Co., Ltd.), 27 December, 1991 (27. 12. 91) (Family: none)	1-8
Y	FEBS LETTERS, Vol. 400, No. 2 (1997) P177-182, particularly P178 2.2	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 December, 1998 (04. 12. 98)	15 December, 1998 (15. 12. 98)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

EXHIBIT C

Clinical Chemistry 50:9
1503–1510 (2004)

Minireview

Biochemistry of Pro-B-Type Natriuretic Peptide-Derived Peptides: The Endocrine Heart Revisited

JENS PETER GOETZE

Background: **Since the discovery of cardiac hormones almost 25 years ago, a vast amount of clinical research has identified the cardiac natriuretic peptides and their precursors as markers of heart failure. It even seems likely that the pro-B-type natriuretic peptide (proBNP)-derived peptides in plasma may become the most frequently measured peptides in the daily diagnosis and control of therapy. In contrast, the biochemistry of the peptides has received less attention.**

Methods: **Published data available on the National Library of Medicine (NLM) were used as the basis for the review.**

Outcome: **This review shows that the present understanding of the biochemistry of peptides is far from complete. In particular, cellular synthesis, including posttranslational precursor maturation, is poorly understood. Moreover, elimination of the precursor fragments is unknown. Elucidation of the molecular heterogeneity of proBNP products will therefore contribute to the understanding of the endocrine heart and may also have important diagnostic consequences. Above all, the different proBNP-derived peptides may not always be equal markers of the same pathophysiologic processes. A different metabolism and peripheral elimination may also impose new and peptide-specific limitations for diagnostic use.**

Conclusions: **It is necessary to focus more on the biology of the proBNP-derived peptides. In turn, new insight into the biochemistry could pave the way for more sensitive and disease-specific assays in the clinical setting.**

It has been more than 40 years since the first anatomical clues to an endocrine function of the heart were reported. Electron microscopy revealed secretory granules in atrial myocytes, which structurally resembled storage granules in peptide-hormone-producing cells *(1, 2)*. It was only in 1981, however, that Adolfo de Bold and his coworkers *(3)* put the endocrine heart to the test and infused extracts of atrial tissue into anesthetized rats. The infusion elicited prompt renal excretion of sodium and water, decreased the blood pressure, and increased the hematocrit. The substance was logically named atrial natriuretic factor. Soon after, this factor was purified and identified as a peptide of 28 amino acid residues *(4, 5)* and was named atrial natriuretic peptide (ANP).[1] This discovery of a new peptide paved the way for the later identification of two different but structurally related peptides in porcine brain: brain natriuretic peptide (BNP) and C-type natriuretic peptide *(6, 7)*. However, BNP was found to be produced mainly in the heart *(8–11)*, and the name "brain natriuretic peptide" now is often replaced with "B-type natriuretic peptide".

The endocrine heart gained further clinical interest when it was reported that patients with congestive heart failure displayed increased plasma concentrations of ANP *(12)*. In parallel, BNP was also shown to circulate in highly increased concentrations in heart failure patients *(13, 14)*. The concept of a plasma marker in heart failure was thereby introduced and has since been intensely pursued, with a strong focus on clinical applications. In addition, N-terminal fragments from the cardiac precursor peptides proANP and proBNP were also found to circulate in plasma and provided new molecular markers for biochemical detection of heart failure *(15, 16)*. At present, proBNP-derived peptides are the most frequently

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Received March 16, 2004; accepted June 21, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.034272

[1] Nonstandard abbreviations: ANP, atrial (A-type) natriuretic peptide; BNP, brain (B-type) natriuretic peptide; proANP and proBNP, propeptides of A- and B-type natriuretic peptide, respectively; and PC, prohormone convertase.

used plasma markers of congestive heart failure. The clinical aspects of proBNP-derived peptides are accordingly frequently and extensively being reviewed *(17–30)*.

Much less is known about the structural biochemistry of the proBNP-derived peptides. For example, cardiac peptide synthesis and secretion remain poorly characterized. Current information on the molecular heterogeneity of proBNP-derived peptides in tissue and plasma suggests an overall simple cellular maturation. However, only the bioactive BNP-32 peptide has been identified, whereas the primary structure of proBNP and its N-terminal fragments remain deduced only from the cDNA sequence. Because cardiac myocytes possess a biosynthetic apparatus including several processing enzymes involved in posttranslational maturation, it seems reasonable to expect that cardiac proBNP maturation may be more complex than initially assumed. In addition, clinical results now imply that plasma concentrations of the different proBNP-derived peptides can vary greatly, which suggests that the myocytes may not always release the peptides on a simple equimolar basis. Finally, there is a peculiar paradox in increased plasma concentrations of cardiac natriuretic peptides in congestive heart failure patients suffering from sodium and water retention. A more comprehensive understanding of the biochemical structures of the molecular forms in plasma may accordingly be of both analytical and biological relevance.

This review will aim at recapitulating the biochemistry of cardiac proBNP-derived peptides. In perspective, future challenges in interpreting results from the clinical use of proBNP-derived peptides may be appreciated.

Nomenclature

A rational nomenclature is essential for the understanding of peptide structure and function. It is also clinically relevant to the physician. If the measured peptide is not readily distinguishable by its name, simple comparisons of measured concentrations from one laboratory to another may confuse and potentially lead to incorrect clinical decisions. Unfortunately, the current nomenclature for the proBNP-derived peptides is far from uniform. The widespread use of abbreviations probably gained popularity because the full peptide name is rather long. However, some suggested abbreviations do not readily identify the measured peptide(s), which clearly should be the primary information within the name. For example, the abbreviation "NT-proBNP" is now most often used for a commercial method and probably refers to measurement of $proBNP_{1-76}$ *(31, 32)*, but the abbreviation does not provide specific information on the primary structure that is actually measured. In particular, it is not clear whether "NT-proBNP" also refers to measurement of the intact precursor. In addition, another used abbreviation, "N-BNP", refers to measurement of both intact proBNP and its N-terminal fragments *(33)*, but this abbreviation may give the incorrect impression that it is the NH_2 terminus of BNP-32 that is being measured. Thus, a rational nomenclature needs to be structurally informative and should give the names in relation to their origin, i.e., with insight in and reference to the posttranslational processing of proBNP (Fig. 1). If this information is not available, then that must clearly be stated. In the following, a uniform nomenclature based on these premises will be used.

Structure of ProBNP

ProBNP is a single, well-defined molecule, a polypeptide that in humans has a length of 108 amino acid residues (Fig. 1). In rodents such as the rat and mouse, the primary structure is slightly shorter but has a similar C-terminal region, which contains the bioactive, receptor-binding sequence (Fig. 2). The mammalian precursor sequences have been deduced from the BNP cDNA sequence that encodes the entire preproBNP molecule *(34–38)*. In addition to proBNP, human preproBNP contains an N-terminal hydrophobic signal peptide of 26 amino acid residues (Fig. 2), but the signal peptide is removed cotranslationally during protein synthesis in the rough endoplasmic reticulum before synthesis of the C-terminal part of the prohormone sequence is completed. It is therefore important to realize that the preproBNP molecule does not exist in real life but is only as a theoretical concept. In contrast, proBNP is likely an existing small protein, the existence of which to date has been indicated by chromatographic studies and sequence-specific immunoassays *(16, 39, 40)*. However, the actual precursor has not been identified on a molecular level, nor have the fragments thereof, apart from the C-terminal BNP-32 peptide itself *(10)*. Thus, it must be emphasized that whenever the primary structures of proBNP-derived peptides are mentioned in the literature, this still refers only to the cDNA-deduced sequence (Fig. 2).

Overall, the proBNP structure appears simple (Fig. 1). In humans, it is divided into two regions after a cleavage

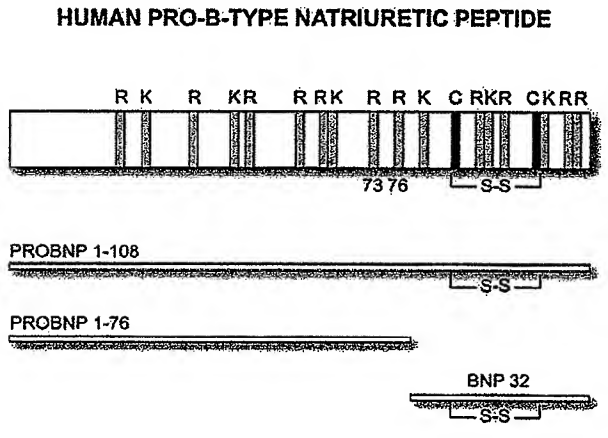

Fig. 1. Schematic presentation of human proBNP and its fragments. The basic amino acid residues are *outlined* (potential cleavage sites) as is the intramolecular disulfide bridge between two cysteinyl residues in the C-terminal region. The C-terminal, bioactive BNP-32 peptide has been fully identified, whereas the complementary $proBNP_{1-76}$ fragment still needs to be purified.

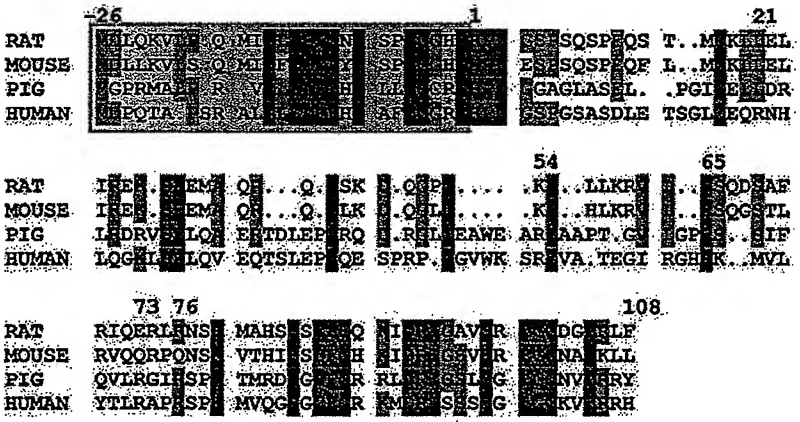

Fig. 2. The primary structure of preproBNP in four mammals.

The *gray box* indicates the signal peptides, which are removed during peptide synthesis. The proBNP sequence in humans thus comprises 108 amino acid residues. *Blue* indicates complete homology among all four species, whereas *red* indicates homology among three of the species. Note that the precursor sequence is evolutionarily preserved in the C-terminal region, which comprises the biologically active natriuretic peptide. In contrast, the cleavage site corresponding to positions 73–76 in the human sequence is not well conserved.

site in positions 73–76 (Arg-Ala-Pro-Arg). The first region is an N-terminal 1–76 fragment, and the second region is the C-terminal BNP-32. In contrast to many other prohormones, proBNP does not contain a third C-terminal region. Rather, the structure mostly resembles the other natriuretic peptides, in which the C-terminal region is a ring formed by a disulfide bond between the cysteinyl residues in positions 86 and 102, respectively (Fig. 2). This disulfide bridge seems essential for BNP receptor binding and biological activity *(41)*. To date, no other proBNP-derived peptides have been identified, which leaves a molecular pattern consisting of only three peptides: intact proBNP, $proBNP_{1-76}$, and $proBNP_{77-108}$; the latter is reasonably named BNP-32 because of its biological effects and molecular size.

Cellular ProBNP Storage

BNP gene expression is a feature of both atrial and ventricular myocytes. In the healthy heart, BNP gene expression occurs mainly in the atria *(42, 43)*. However, ventricular BNP gene expression is up-regulated in diseases that affect the ventricles, such as heart failure *(44)*. This observation may have given rise to the frequent but incorrect statement that BNP is a ventricular hormone. Atrial and ventricular myocytes differ considerably with respect to their endocrine apparatus, and it is reasonable to expect key differences in peptide storage and secretion. As mentioned, it is a well-established fact that atrial myocytes contain secretory granules for peptide storage, which led to the primary hypothesis about the endocrine heart *(1, 2)*. Importantly, atrial granules store both intact proBNP and cleaved products, i.e., bioactive BNP-32. In contrast, ventricular myocytes in the healthy heart do not seem to produce these granules, and do not contain proBNP-derived peptides *(43, 45)*. On the other hand, there have been some reports on both secretory granules and proBNP-derived peptides in ventricular myocytes from diseased hearts *(45–47)*. Thus, the ventricular myocytes not only up-regulate the BNP gene but also seem to differentiate with respect to the biosynthetic apparatus. Therefore, the cellular storage and secretion of proBNP-derived peptides are complex, and the prevailing concept of regulated atrial secretion and constitutive-like ventricular release should still be a strong issue in basic research. Development of biologically relevant in vitro models would be most valuable for such studies. Finally, other cells within the heart also express the BNP gene: cardiac fibroblasts were recently shown to produce and release BNP-32 *(48)*, Moreover, the coronary vasculature also expresses the BNP gene, at least in coronary atherosclerosis *(49)*. Clearly, the precise proBNP storage and secretion from these cells needs to be explored.

Processing of ProBNP

Cardiac processing of proBNP is still poorly characterized. One major reason is the troublesome lack of useful in vitro cellular models *(42)*. Although neonatal atrial myocytes can be cultured for short periods of time, they do not resemble the differentiated atrial or ventricular myocytes. Moreover, only a few sequence-specific assays have been developed for the various regions within the proBNP molecule, apart from the NH_2 and COOH termini. Accordingly, the available information on posttranslational proBNP processing is partially based on indirect observations, i.e., from chromatographic studies without precise calibrators.

At first, proBNP was suggested to be cleaved by the ubiquitous endoprotease furin because the genes for both furin and BNP are expressed in cardiac myocytes of the diseased heart *(50, 51)*. In addition, the Arg-X-X-Arg motif in positions 73–76 in proBNP has been shown to be a target for furin-mediated cleavage. In fact, processing of proBNP can be blocked in vitro by inhibition of furin *(51)*, and furin has been shown to be critical in the processing of the structurally related peptide, pro-C-type natriuretic peptide *(52)*. Recently, a novel protease named corin was identified from human heart cDNA *(53, 54)*. Corin is a serine protease that can cleave both proANP and proBNP in vitro; presumably at a similar cleavage site *(55, 56)*. Moreover, corin contains a transmembrane domain, is located within the cell membrane, and has been suggested to cleave the precursors on secretion *(56)*. The enzymatic

activity does not require the transmembrane domain, however, because a mutant soluble form is also capable of processing proANP *(57)*. At present therefore, corin seems to be a candidate for cardiac proBNP maturation and may be involved in the generation of $proBNP_{1-76}$ and BNP-32. On the other hand, no reported study has elucidated exactly where corin cleaves the primary proBNP structure. Moreover, atrial processing of proANP and proBNP differs: isolated atrial granules have been shown to contain proANP and BNP-32 *(58)*. Thus, corin activity does not fully explain the posttranslational processing of cardiac natriuretic peptides.

A well-established family of intracellular processing enzymes involved in prohormone maturation is the prohormone convertases (PCs). In addition to the above-mentioned furin, the subtilisin-like endoproteases PC1 and PC2 are also produced in rat heart *(59, 60)*, and PC1 production has recently been shown in healthy and diseased human cardiac tissue *(61)*. Interestingly, atrial myocytes transfected with an adenoviral vector that expresses PC1 can process proANP to both mature ANP and to a truncated form *(62)*. Although the precise cleavage site was not established and the processing capacity was very inefficient, this singular report does raise the possibility that other endoproteases may be involved in the posttranslational maturation of proBNP. Importantly, PC1 is active in secretory granules and could therefore be a feature of atrial proBNP processing. We are currently examining the cellular processing of proBNP by expressing the human BNP gene in endocrine cells. Preliminary results indicate that cells that produce PC1 do process proBNP, which supports the idea of PC1 as a candidate in cardiac posttranslational maturation of proBNP. Unfortunately, there is still no information on cardiac proBNP processing at other potential cleavage sites in the precursor (Fig. 1). Thus, there is a need for molecular identification of proBNP-derived peptides in cardiac tissue, which would give further insight into the cardiac processing of proBNP. Moreover, such information could be useful for designing new diagnostic immunoassays.

ProBNP-Derived Peptides in Plasma

ProBNP-derived peptides are secreted by the cardiac myocytes and circulate in plasma. Their molecular heterogeneity has been examined by chromatography and sequence-specific immunoassays. It is established that BNP-32 is secreted directly from the heart *(63)* and circulates without binding to plasma proteins. However, synthetic BNP-32 is trimmed when incubated in whole blood, which generates a dominant BNP form lacking the two N-terminal amino acid residues (Ser-Pro; Fig. 2) *(64)*. Interestingly, this X-Pro motif is a known cleavage site in chemokines, cytokines, and other signal peptides *(65)*, and enzymatic removal of the two N-terminal X-Pro residues has been demonstrated in serum *(66, 67)* and, recently, in intracellular vesicles *(68, 69)*. It therefore seems likely that endogenous BNP-32 may undergo N-terminal trimming by an amino-dipeptidase. Of note, although this N-terminal region does not seem critical for receptor binding and biological activity *(41)*, enzymatic cleavage at the NH_2 terminus may be critical when choosing epitopes for antibody production and immunoassay design.

In circulation, BNP-32 is generally believed to be metabolized by a membrane-bound endopeptidase (NEP 24.11) as well as by receptor-mediated cellular uptake. The metabolic half-life of BNP-32 has been reported to be 13–20 min *(70, 71)*. BNP-32 is also present in urine *(72, 73)*, but the precise mechanism of renal excretion is not fully understood. We recently reported minor BNP-32 clearance by the liver, which is not significantly altered in patients with cirrhosis *(74)*. More studies are required, however, to elucidate the precise sites of metabolism. In turn, such studies could identify disease mechanisms that may influence, and possibly even limit, the diagnostic use of plasma measurements.

In addition to BNP-32, other proBNP fragments circulate in plasma *(16)*. These fragments are commonly referred to as "N-terminal proBNP", but the molecular heterogeneity also includes the intact precursor, in particular in heart failure patients *(16, 39, 40, 75)*. Cardiac secretion of proBNP and its N-terminal fragments has been demonstrated by blood sampling from the coronary sinus *(76, 77)*. The precise molar ratio of secreted $proBNP_{1-76}$ to intact proBNP has not, however, been clarified, which reflects a lack of reliable region-specific assays as well as difficulties in the chromatographic separation procedures. However, one immunoassay using antibodies raised against the COOH terminus of $proBNP_{1-76}$ has been developed *(78)*. This assay measured $proBNP_{1-76}$ in plasma from heart failure patients, but the assay did not detect the fragment in cardiac tissue extracts. Moreover, it was suggested that intact proBNP can be processed to N- and C-terminal fragments in serum, but not in whole blood or plasma. Most proBNP, however, is processed before reaching the circulation *(78)*. We have recently used a somewhat different strategy that bypasses some of these inherent problems *(40)*. When we incubate plasma with trypsin before analytical measurement, the trypsin cleaves endogenous proBNP at monobasic residues to release a small $proBNP_{1-21}$ fragment (Fig. 1). In this way, both intact proBNP and $proBNP_{1-76}$ (and other possible N-terminal fragments) are processed into the same analyte, which subsequently is measured with a conventional RIA specific for the N-terminal epitope of proBNP. This processing-independent assay, designated PIA, may provide a more general approach to quantify the total amount of precursor-derived peptides in plasma *(79)*.

Another troublesome dilemma is the unknown metabolism of proBNP and its N-terminal fragments. Apart from a single study in sheep, which suggested a longer half-life of proBNP than BNP in circulation *(80)*, there are no pharmacokinetic data available on the elimination of proBNP. This type of information will be critical for the

full interpretation of plasma measurements as well as for identification of the possible role of other organs on the circulating concentrations. Thus, the pharmacokinetics and organ clearance should be a focus for future research.

ProBNP and Oligomerization

The presence of "high-molecular-weight" proBNP in circulation has recently been suggested *(81)*. In that report, immunoreactive N-terminal proBNP in plasma was shown to elute at a position corresponding to a much higher molecular weight than expected by chromatography, and a small synthetic proBNP-derived peptide in buffer also eluted at a position corresponding to a molecule approximately three times larger than its molecular weight. Surprisingly, the phenomenon was completely abolished in a denaturing buffer. The presence of a leucine zipper-like motif in the proBNP sequence (Fig. 2) led the authors to suggest that proBNP and its N-terminal fragments oligomerize *(81)*. In addition, the immunoreactivity of high-molecular-weight BNP in heart failure plasma has also been reported *(64, 75)*. We also have encountered this phenomenon, which extends even to proBNP in extracts of atrial tissue (Fig. 3). However, synthetic $proBNP_{1-39}$ with the leucine zipper-like motif substituted with alanyl residues also elutes as a molecule three times larger than the expected size, and the elution position can be reversed to the expected with use of denaturing conditions (Goetze et al., unpublished data). Thus, although proBNP and its N-terminal fragments seem to associate to something in cardiac tissue and plasma, the underlying mechanism still needs to be determined. It is nevertheless important to emphasize that such oligomerization may have a major influence on antibody detection and assay performance.

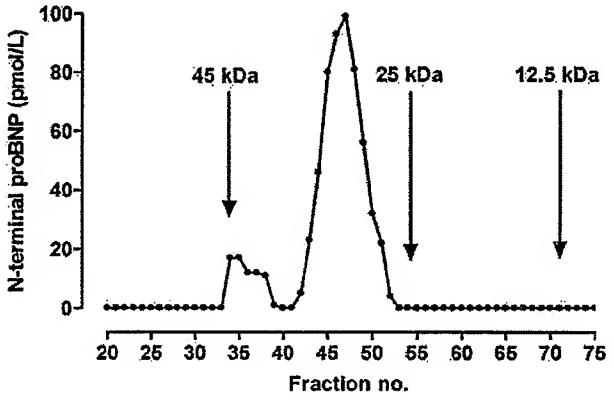

Fig. 3. Chromatographic profile of proBNP in human atrial tissue.

Neutral tissue extract was subjected to size-exclusion HPLC (Superdex 75; Pharmacia) and eluted with a physiologic buffer. Molecular size calibrators were eluted in a separate run. The proBNP immunoreactivity eluted in positions approximately three and four times higher than the theoretical molecular mass of intact proBNP (11 900 Da). The elution was reversed to the expected position corresponding to the intact precursor when denaturing conditions were used (data not shown).

Endocrine Paradox in Heart Failure

Heart failure patients have highly increased plasma concentrations of BNP-32 *(21)* with marked up-regulation of BNP gene expression and subsequent high plasma concentrations of proBNP-derived peptides; it therefore seems reasonable to expect increased natriuresis. However, the opposite is the common clinical situation, with heart failure patients suffering from congestion, sodium retention, and edema. Although heart failure is a complex condition, with both activation and inhibition of nervous and hormonal systems, the paradoxical lack of ANP and BNP effects is still compelling. Of note, heart failure patients respond as expected to intravenous administration of synthetic ANP and BNP. An explanation for this endocrine paradox may be sought in the biochemistry of cardiac natriuretic peptides *(82)*. As mentioned, both ANP and BNP are synthesized as prohormones and require posttranslational processing to release the highly potent natriuretic peptides. Importantly, the unprocessed precursors may also possess some bioactivity, which is underscored by the natriuretic effect elicited by infusion of atrial tissue homogenates containing mostly proANP. Prohormone maturation, however, seems mostly a feature of atrial peptide synthesis. In support of this, the proposed enzymes involved in proBNP maturation are produced predominantly in atrial myocytes *(53, 60–62)*, and the ventricular myocytes do not, at least to the same extent, contain secretory granules for peptide storage and maturation. The posttranslational processing of ventricular proBNP may consequently not always be efficient in the production of potent natriuretic hormones. Immunoassays may nevertheless still detect the intact prohormone and therefore not reflect the actual bioactivity of the secreted peptides. In agreement with this suggestion, patients with congestive heart failure have increased plasma concentrations of immunoreactive N-terminal proBNP, and this immunoreactive material seems to also contain a polypeptide corresponding to the intact precursor *(16, 39, 40)*. Thus, the congestion in heart failure patients may not be ameliorated by the secreted natriuretic peptides. Moreover, there may occasionally be large individual differences in the ability of the heart tissue to mature the precursor peptides, which could help explain why some heart failure patients suffer from severe congestion and edema, whereas other patients have much less congestion. Although this suggestion is still hypothetical, it could be worthwhile to focus more on the molar differences among proBNP-derived peptides in plasma. For example, patients with highly increased proBNP and N-terminal fragments but only modestly increased BNP-32 concentrations in plasma could be compared with patients with high BNP-32 concentrations. Finally, precursor peptides in plasma may be a future target for drug treatment because circulating precursors perhaps could be activated to mature BNP-32 and, thus, to potent hormonal activity.

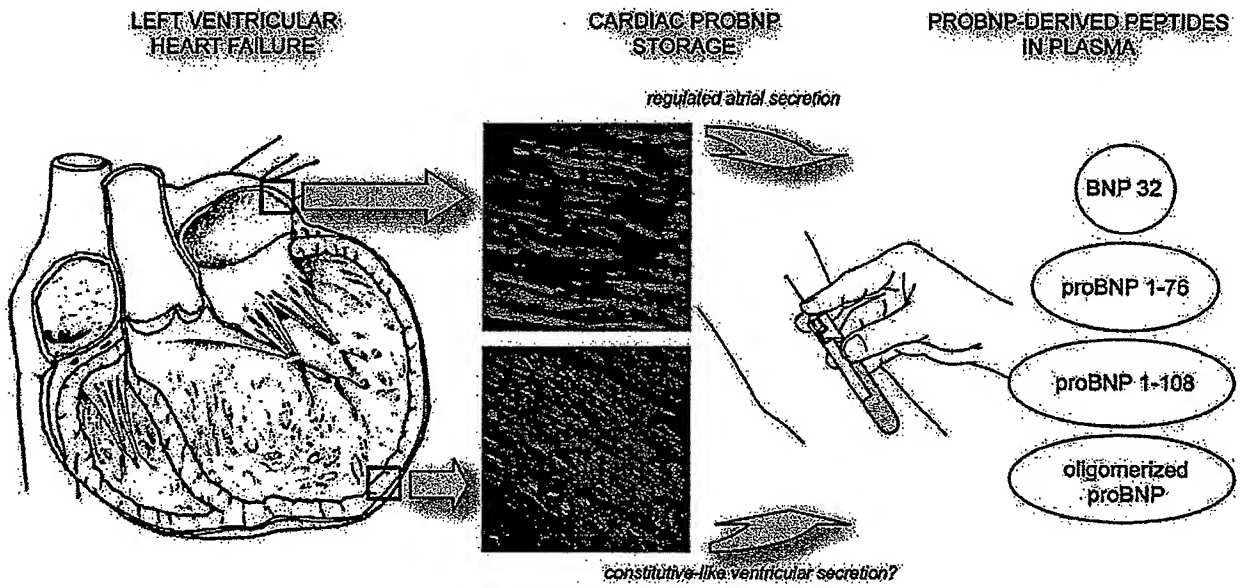

Fig. 4. Schematic presentation of the proBNP-derived peptides in heart failure.

Dilation of the left ventricle increases cardiac BNP gene expression. Confocal microscopy of human atrial and ventricular cardiac tissue shows the cellular storage of proBNP, detected with antiserum to N-terminal proBNP. Atrial peptide secretion is regulated, whereas ventricular peptide release is not clarified in heart failure. The BNP-32, proBNP$_{1-76}$, and intact proBNP forms circulate in peripheral plasma, together with oligomerized proBNP.

Conclusions

Since the principal discovery of the cardiac hormones almost 25 years ago, a tremendous amount of research has identified the proBNP-derived peptides as useful plasma markers in heart failure (Fig. 4). It even seems likely that these peptides may become the most frequently measured peptides in the clinical routine. In contrast, our present understanding of the structural biochemistry is still far from complete. In particular, cellular synthesis, including posttranslational maturation and metabolism of the peptides, is poorly characterized. Further elucidation of the molecular heterogeneity could provide important biological insight into the endocrine heart and could likely have important diagnostic consequences. Above all, the different proBNP-derived peptides may not always be equal markers of the same pathophysiologic processes. In addition, differences in elimination may introduce new boundaries for diagnostic use. With the need for markers in heart failure being firmly documented, it now seems important to focus more on the biology of the proBNP-derived peptides. In turn, new insights into the structural biochemistry could pave the way for better and more disease-specific measurements in the clinical setting.

I am grateful to Professor Jens F. Rehfeld for his continuous encouragement and inspiration in my work with peptide hormones and their precursors.

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EXHIBIT D

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Gene 292 (2002) 183–190

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

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Cloning and characterization of feline brain natriuretic peptide

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Received 2 February 2002; accepted 7 May 2002
Received by J. Widom

Abstract

Brain (B-type) natriuretic peptide (BNP) is a cardiac hormone involved in regulation of fluid balance and blood pressure homeostasis of mammalian species. BNP sequence is species-specific and considered to be a significant prognostic and diagnostic marker for cardiac dysfunction. Using conventional polymerase chain reaction and amplification of cDNA 3′- and 5′-ends, a total of 1500 nucleotides encompassing the entire feline BNP gene were characterized. The feline BNP gene is organized in three exons separated by two introns. The complete transcript of 736 nucleotides was characterized, including 396 nucleotides encoding feline preproBNP. The preproBNP consisted of a signal peptide of 26 amino acids and a proBNP of 106 residues. The predicted mature BNP comprised 35 amino acids with likely 26- and 29-aa isomers, including a histidine residue at the C-terminus. Based on the similarity of BNP prepropeptide sequences, a phylogenetic relationship is presented for mammalian species including human, cat, cattle, dog, mouse, rat, sheep and swine.

Keywords: Brain (B-type) natriuretic peptide; cardiac hormone; *Felis catus*

1. Introduction

Brain (B-type) natriuretic peptide (BNP) was first discovered in porcine brain (Maekawa et al., 1988). Later studies have revealed that BNP is highly expressed in cardiac myocytes and mainly distributed in heart tissue (Hasegawa et al., 1997; Magga et al., 1998; Saito et al., 1989). BNP is a member of the cardiac natriuretic hormone family. Other members of this family include atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and other peptides derived from the N-terminus of the proANP and proBNP proteins. These natriuretic peptides are structurally similar but genetically distinct. As a group of cardiac hormones, they are involved in regulation of fluid balance and blood pressure homeostasis in mammalian species (Samson, 2000). BNP and ANP are of myocardial cell origin, whereas CNP is of endothelial and renal epithelial cell origin (Mukoyama et al., 1991; Stingo et al., 1992). Within the cardiac tissues, BNP is predominantly of ventricular origin, in contrast to ANP of atrial origin (Yashu et al., 1994; Yoshimura et al., 1993). Enhanced BNP production is present in chronic congestive heart failure, or hypertrophy (Kohno et al., 1992; Mukoyama et al., 1991). BNP is considered to be a significant prognostic and diagnostic marker for various cardiac dysfunctions, and it may also have therapeutic potential for heart failure (Chen and Burnett, 1999; Grantham et al., 1997; Yamamoto et al., 1996). The nucleotide and amino acid sequences of BNP have been identified for several mammalian species, including human (*Homo sapiens*), cattle (*Bos taurus*), dog (*Canis familiaris*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), sheep (*Ovis aries*) and swine (*Sus scrofa*) (Aitken et al., 1999; Kojima et al., 1989; Maekawa et al., 1988; Nguyen et al., 1989; Ogawa et al., 1994; Seilhamer et al., 1989; Sudoh et al., 1988). These sequences have provided the basis for peptide synthesis and antibody production against various BNP species. Numerous commercial antibodies and antibody-based assays are currently available. Among the reported cardiac natriuretic hormones, each peptide has different production and secretion patterns. Therefore, the pathophysiological and clinical values vary for the assay development for each hormone. Unlike the universal nature of ANP antibodies, antibodies of BNP are species-specific.

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain (B-type) natriuretic peptide; CNP, C-type natriuretic peptide; RACE, rapid amplification of cDNA ends

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0378-1119/02/$ - see front matter
PII: S0378-1119(02)00676-5

It is believed that BNP provides a more efficient clinical tool compared to the other cardiac natriuretic hormones (Clerio et al., 2000). The domestic cat (*Felis catus*) is frequently diagnosed to have hypertrophic cardiomyopathy. A BNP gene-based assay is in demand by veterinary clinicians as a valuable biochemical marker. However, the feline BNP gene has not been characterized and the BNP amino acid sequences are unknown. The objective of this study was to sequence the feline BNP gene and identify the BNP amino acid sequence. This paper presents the feline BNP gene sequence, its complete messenger RNA (mRNA) transcript, and the deduced amino acid sequence.

2. Materials and methods

2.1. Oligonucleotide primers

Nucleotide sequences of oligonucleotide primers used in this study are listed in Table 1. All BNP primers designed in this study were synthesized in the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign, Urbana, IL). Adaptor primers used in 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE from Gibco BRL (Grand Island, NY) and CLONTECH Laboratories, Inc. (Palo Alto, CA) were applied.

2.2. Genomic DNA preparations

Fresh feline blood was collected in tubes containing ethylenediaminetetra-acetic acid (EDTA) anticoagulant and suspended in a RBC (red blood cell) lysis buffer. White cells were collected by centrifugation and genomic DNA was extracted using a Wizard Genomic DNA purification procedure according to the manufacturer's instructions (Promega, Madison, WI).

2.3. Total RNA preparations

Cardiac tissues from cat atrium and ventricle were sampled within 20 min of dissection. The sampled tissues were cut into 1 cm^2 pieces and emerged in pre-cooled RNALater (Ambion, Inc. Austin, TX) separately. The submerged samples were equilibrated at 4°C for 24 h and then stored at −20°C until use. One hundred mg of each sample heart tissue was ground into fine powder in liquid nitrogen and homogenized in a lysis buffer using a high-speed mechanical homogenizor. The residual heart tissue was discarded by centrifugation at low speed. The supernatant was used to isolate the total RNA using a fiber filter-based procedure (Ambion, Inc., Austin, TX). Total RNA integrity was monitored by denaturing agarose gel electrophoresis. RNA concentrations and purity were measured by spectrophotometry.

Table 1
Nucleotide sequences of oligonucleotide primers used in this study

Identification	Sequence 5′–3′	Length
BNPF1	CTCCTGTTCTTGCACCTGTC	20-mer
BNPF3	CGAAGCGTCCGCAATACAGGA	21-mer
BNPF9	CTTGCACCTGTCGCCACTA	19-mer
BNPR2	CAGTTGCAGCCCAGGCCACT	20-mer
BNPR4	TCTCAGCACGTTGCAGCCCAGGC	23-mer
BNPR6	AATTGCTTCAAAGGGGCTCA	20-mer
BNPR10	TGAAACTGTGTCCCGCAGA	19-mer

2.4. cDNA synthesis

For conventional polymerase chain reaction (PCR), cDNA was synthesized using Superscript II and primed with oligo (dT) followed by a standard procedure (Gibco, Grand Island, NY). An adapter primer was used to synthesize the first strand of cDNA for 3′-RACE. The reaction consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM adapter primer, 500 μM each of dATP, dCTP, dGTP and dTTP with 1 μg of total RNA. The cDNA template was treated with RNase H after the reaction. For 5′-RACE, SMART (switching mechanism at 5′ end of RNA transcript) PCR was used (Chenchik et al., 1998). Moloney Murine Leukemia Virus reverse transcriptase (Ambion, Inc. Austin, TX) was used and primed with the SMART II oligonucleotide and 5′-CDS (5′-RACE codons) primer (CLONTECH Laboratories, Inc., Palo Alto, CA) to generate a complete cDNA copy of the original RNA. The final composition of the reaction consisted of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, and 1 μM primer with 180–360 ng of total RNA from atrium or ventricle. The reactions were treated with RNase H after termination of the reaction.

2.5. PCR and amplification of cDNA ends

PCR amplifications were carried out in a 25 or 50 μl volume containing *Taq* polymerase and 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 1 × PCR buffer. The profile for the RACE procedures were optimized as previously described (Frohman, 1990). For 3′-RACE, the target cDNA was amplified using primer BNPF3 designed from a cat sequence (AF253495) as a forward primer paired with a reverse adaptor primer. The final composition of the reaction consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM each of the primers, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.04 U/μl of *Taq* DNA polymerase with cDNA as template. The thermal profile for the 3′-RACE reaction comprised initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 45 s.

The first strand product was diluted 3–10 times using Tricine-EDTA buffer (10 mM Tricine-KOH (pH 8.5) and 1.0 mM EDTA). Based on the sequence obtained from the 3′-RACE, a cat specific primer BNPR4 was designed as a reverse primer for a 5′-RACE reaction. The reaction concentration for the 5′-RACE was the same as that used for the 3′-

RACE with the exception of 20 μM of the universal long primer, 100 μM of short primer and 200 μm of BNPR4. The thermal profile for the 5′-RACE included an initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 3 min, and a final extension at 72°C for 5 min.

2.6. Subcloning and DNA sequencing

PCR products were purified using the Wizard DNA purification system (Promega, Madison, WI), ligated into a pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109 high efficiency competent cells to maintain the recombinant DNA. Positive recombinant cells were selected using LB agar with ampicilin/IPTG/X-gal. DNA restriction digest by *Pst*I and sequencing analysis were conducted to confirm clone identity.

Plasmid DNA with the desired insert was isolated using acetyl-trimethylammonium bromide miniprep method and column purified (Promega, Madison, WI). DNA sequences were determined by sequencing reactions primed with primer T7, SP6 or various cat BNP gene specific primers designed in this study (ACGT, Inc. Northbrook, IL). Overlapping sequences were confirmed by sequence analysis from both priming directions. Mismatched sequences were verified by sequencing overlapped subclones and manual editing of the chromatograms. Intron/exon boundaries were identified by comparison of sequences obtained from mRNA and genomic DNA. Sequences were analyzed by comparison with known genes of other species using the BLAST algorithm (Altschul et al., 1990).

2.7. Northern blot

RNA samples were denatured and separated by electrophoresis in a 1% denaturing agarose gel. Following electrophoresis, the RNA was transferred to Bright Star-Plus, a positively charged nylon membrane (Ambion, Inc., Austin, TX). A BNP-PCR product of 288-bp was purified and random-primed labeled with ^{32}P (Amersham Pharmacia Biotech, Piscataway, NJ) as a probe for hybridization. After prehybridization at 42°C, the labeled probe was denatured and transferred on to the membrane. Hybridization was carried out overnight at 42°C. Following hybridization, the membrane was washed and exposed to X-ray film. The size of the BNP mRNA transcripts was determined as previously described (Solter et al., 2000).

2.8. Phylogenetic relationship

PreproBNP sequence of the cat was aligned with seven other known mammalian species, including human, cattle, dog, mouse, rat, sheep and swine for comparison of similarities. Conserved sequences were identified and phylogenetic relationships among the species were examined as previously described (Thompson et al., 1994).

3. Results

3.1. Cloning of BNP gene

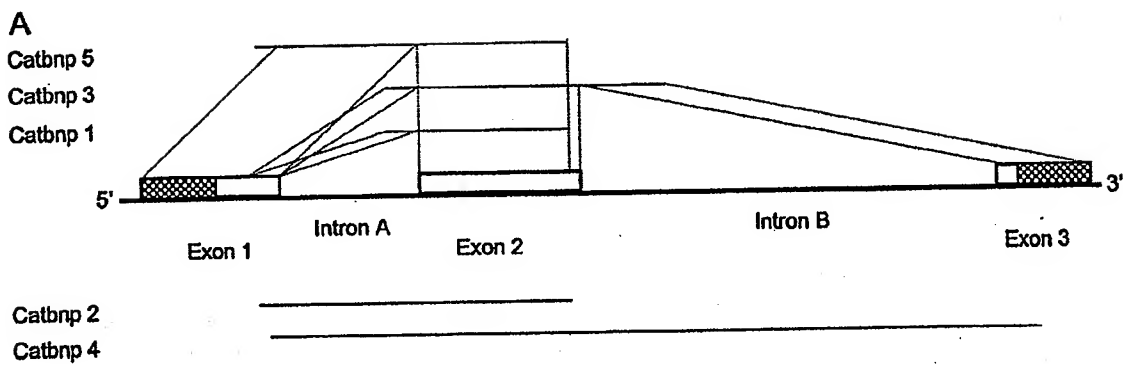
Primers BNPF1 and BNPR2 were designed based on consensus BNP gene sequences of other mammalian species. After verification by *Pst*I restriction digest and sequence analysis, a 350-bp fragment amplified from feline cDNA was identified as a portion of feline BNP coding sequence (Fig. 1A). A fragment of 580-bp, clone Catbnp2, was obtained using the same pair of primers and genomic DNA as template (Fig. 1A). Comparison of the sequences showed that a 236-bp intron existed between exons 1 and 2 (intron A). The sequence obtained from this mRNA transcript contained a portion of exon 1 and the majority of exon 2.

The characterization of the cat BNP was further expanded at the 3′-end based on the mid-section sequences. A 500-bp fragment was obtained by 3′-RACE using RNA samples isolated from atrium (Fig. 2A). Sequence analysis revealed that this 500-bp cDNA clone, Catbnp3, contained a complete 3′-end sequence, including the entire mature BNP peptide (Fig. 1A). It included the AT-rich sequences and the polyadenylation signal followed by a poly-A tail (Fig. 1B). A subsequently obtained 1.2 kb fragment of genomic DNA clone produced by primers BNP3 and BNP6, Catbnp4, was found to include the entirety of intron A, intron B, and exon 2 (Fig. 1A). BNPR6 was a reverse primer designed from the previously identified sequences of clone Catbnp3. Comparative analysis of the sequences derived from cDNA and genomic DNA indicated a third exon of 14 bp, encoding for cat BNP C-terminal, and intron B of 574-bp between exons 2 and 3.

For the 5′-RACE, clone Catbnp5 contained a 480-bp target fragment (Figs. 1A and 2B), including 5′ region exon 1 and a putative promoter region (Fig. 1B). Thus, a complete mRNA transcript was identified consisting of 736 nucleotides with a 396-bp coding region. Northern blot hybridization showed a feline BNP mRNA transcript of about 0.7 kb in size, in agreement with the results of the sequence analysis.

3.2. Genomic structure of BNP

A total of 1500-bp nucleotides were obtained for feline BNP gene. Complete cat BNP mRNA and gene sequences were deposited at GenBank (Accession nos. AF425738 and AF253495). The deduced amino acid sequence had a total of 132 amino acids for feline preproBNP (Fig. 1B). The signal peptide contained 26 amino acid residues, followed by 106 amino acids of proBNP. A mature feline BNP form was assumed to comprise 35 amino acid residues at the C-terminus (Fig. 1B). The cat BNP gene was organized with two introns separating the three exons (Fig. 1B). Splice donor and acceptor consensus sequences were identified at the exon/intron boundaries. A putative cap site appeared 43 bp 5′ upstream to the ATG translation start codon. AT-



B

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ACGCGGGGAGAGCGGCCGCCGGCCTCTAGCAGGTGAGACGTGACCCGGAACACAGCGCAG
CGGCAGGGGCAACGATCTCTTTCTCTCCGGTGACATGGACCCCAAGACGGCGCTGCTCCG
                                  M  D  P  K  T  A  L  L  R

GGCCCTCCTGCTCCTCCTGTTCTTGCACCTGTCGCCACTAGGAGGTCGCTCCCACCCGCT
 A  L  L  L  L  L  F  L  H  L  S  P  L  G  G  R  S  H  P  L

GGGCGGCCCCGGCCCCGCCTCGGAAGCGTCCGCAATACAGGTGAGAGAGCTTCAGCCTGC
 G  G  P  G  P  A  S  E  A  S  A  I  Q

GAAAGGGCTGCTGTGGGTAGCAGCAGTTAAACGGGTCCTGAGCTATTGTCCCAAAAGGTC
CTCAGCCCCTCTAGCGAATTAGTGATAAGGGATTAGAAAGCAACCGGATGGGGTGCCAGG
ACCCCTACCTAAGGTGGTTGGTTCATCTCAGCTCCGTCTAGGGCGGTTTGGCCAGGTGCG
GGTTGCTGAGGCTGGGGCTGTCCCCACGCATTGCAGGAGCTGCTGGACGGTCTGCGGGAC
                                  E  L  L  D  G  L  R  D

ACAGTTTCAGAGCTGCAGGAAGCCCAGATGGCCCTGGGACCCCTCCAGCAGGGTCACAGC
T  V  S  E  L  Q  E  A  Q  M  A  L  G  P  L  Q  Q  G  H  S

CCCGCGGAATCCTGGGAGGCCCAGGAGGAACCCCCTGCGCGGGTCCTTGCGCCCCATGAC
P  A  E  S  W  E  A  Q  E  E  P  P  A  R  V  L  A  P  H  D

AATGTCCTCCGGGCCCTGAGACGACTAGGCAGTTCCAAGATGATGCGTGATTCAAGGTGC
N  V  L  R  A  L  R  R  L  G  S  S  K  M  M  R  D  S  R  C

TTTGGCCGGAGGCTGGACCGGATTGGCTCCCTCAGTGGCCTGGGCTGCAACGGTAAGCCC
F  G  R  R  L  D  R  I  G  S  L  S  G  L  G  C  N  V

CCTCCCTGCCAGCTTGATCCCCCTGCCCCACTCCTATGGGTCCCACCATCGGAACCGCTT
CTGGGTTTGTTGTCTCTGGGAACCAATTCTGAGAAAAAGACACTTGGGCTTTACTCTCTC
TTGCTGACAGTGCTTAAGGCCAAGGGGCGCCTTTCTGGAAATATTAAATGTGGACATCAT
TCGTTACTATGTCCACGAGTCCCTACCCACCTTCTCTTGCCTCTCTGGCCAAAAGGGGCA
GAGTCGCCTTAGGATGGAATCCAGTCATTCTCTGGCTGCATCTCCTTGGAGCAGAAAGGG
TCCTAAACGTTTTCCTCCTGGTTTCCCCCAGCCTGTCTATAGCCTCAAAGGCAGAGGACG
GGATCACGAGGACAACAATAATTGCCAGTTTACAGATGAGGAGACAGAGGTCCAGAGAGT
GGATTATTCTAAAGCCTCAAACACCCAGATCAGCCGAGGTTGGGGGTGGGGGGTGGTGGC
AGGGATCACACCCAGTGTGGGGAAGCTCAGATCCTGCCTCAGTTTCCCACCTGACGCCGT
CATCACCCTTTCTCCCCCCCACACAGTGCTGAGAAGGCATTAAGAGGAAGTGCTGATGCA
                              L  R  R  H

GACATCTGCGTCTGATTCTCCATCAACCCCCTGAGCCCCTTTGAAGCAATTCTTCTTTAT
TTATTTTTATTTATTTATTTATTGGGATTGTTTTATATAAGATGATTCTTATCTCTGAGC
ACCCATTTTCCACGGTGAAATAAAGTCAACATTTTATCTTCAAAAAAAAAAAAAAAAAAA
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Fig. 1. Gene structure and coding regions of cat BNP preproBNP. Panel A: Schematic representation of cat BNP gene organization and cDNA clones (odd numbered Catbnp clones) and genomic DNA clones (even numbered Catbnp clones). Coding regions are boxed and the untranslated regions shaded. Panel B: Nucleotide sequences and deduced amino acid sequences of the cat BNP gene. Translated exons are bolded, the stop codon is bold-underlined, splice donor and acceptor consensus sequences are dot-underlined, the AT-rich region is dash-underlined, the polyadenylation signal is double underlined and the putative cap region is thin-underlined. The deduced amino acid sequences are displayed under the corresponding nucleotide sequences, and the mature cat BNP sequences are underlined.

rich sequences were located at the 3′-untranslated region and followed by a typical polyadenylation signal, 17 bp upstream to the poly-A tail. Exon 1 consisted of 126 bp, encoded the signal peptide and 16 amino acids of the N-terminal peptide. Introns A and B were 236 and 574 bp in length, respectively. Exon 2 consisted of 256 bp, encoded

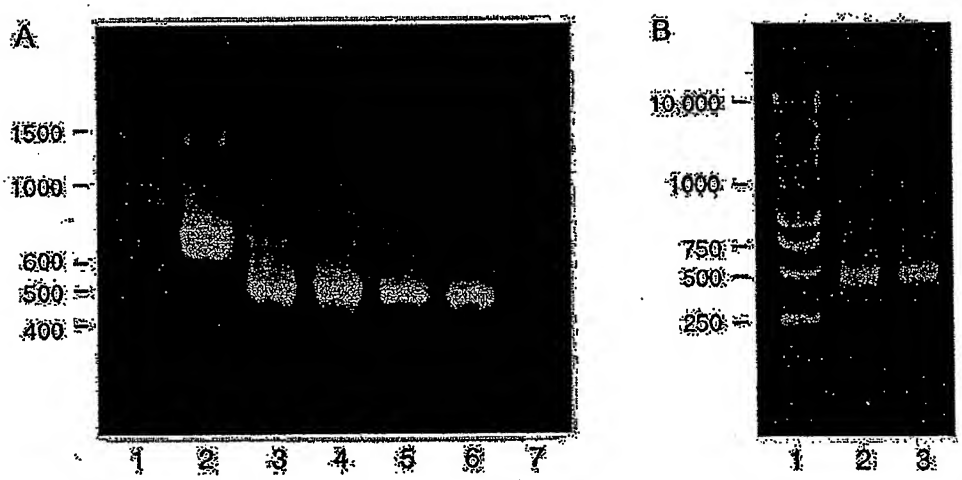

Fig. 2. A 1.2% agarose TBE gel showing target products of 3′-RACE (A); and 5′-RACE (B) -PCR. Lane 1 in panel A is a molecular marker (bp), lane 2, a control product of 720 bp, lanes 3–6, cDNA templates derived from atrium RNA samples, and lane 7 is a ventricle RNA sample of 500 bp products. In panel B, lane 1 is a molecular marker (bp), lane 2, cDNA derived from an atrium RNA sample, and lane 3, a ventricle sample, showing products of 480 bp fragments.

the remaining N-terminal-prepropeptide and a majority of the mature peptide at the C-terminus. Exon 3 contained 14 bp, encoding two bases of slicing codon for a valine and the four C-terminal end amino acid residues of the mature peptide, followed by a TAA stop codon.

3.3. Comparison of preproBNP among mammalian species

Comparison of cat BNP prepropeptide sequence with other known species, including human, cattle, dog, mouse, rat, sheep and swine showed significant sequence similarity in the entire preproBNP region (Fig. 3). There were highly conserved regions in BNP sequences among these species, particularly in the mature BNP encoding region (Fig. 3). Feline mature BNP showed a histidine residue identical to that of human at the COOH-terminus. Based on the similarity of preproBNP sequences, mouse and rat were distinctly separated from other mammalian species (Fig. 4). Cattle, sheep and swine formed another closely related group. Cat appeared to be closely related with dog. In contrast, human was a distinct group as compared to the other species.

4. Discussion

The present study obtained feline BNP gene sequence consisting of three exons separated by two introns with regulatory element coding regions. This gene organization

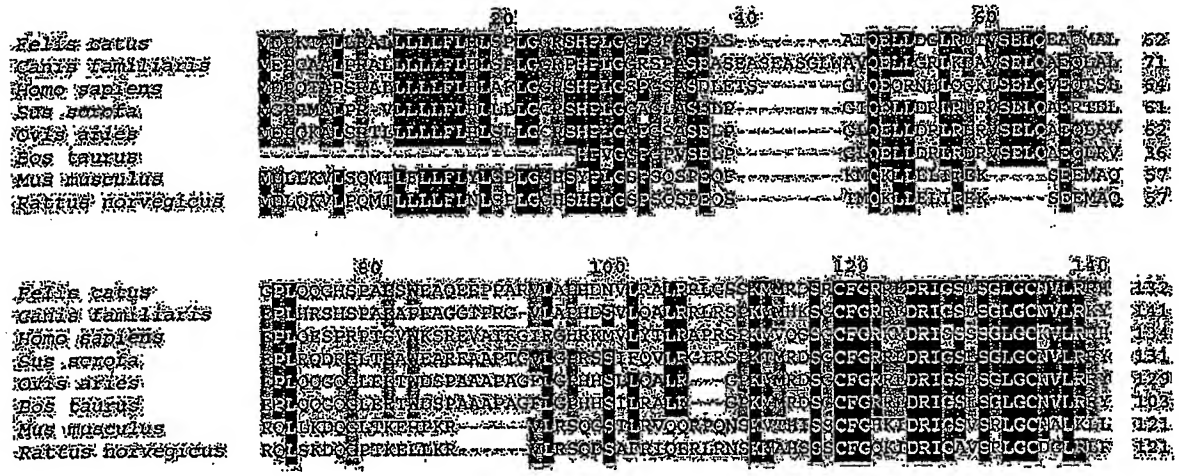

Fig. 3. Comparisons of preproBNP amino acid sequences of cat, human, cattle, dog, mouse, rat, sheep and swine, showing similarities among species. Totally conserved amino acids are dark shaded, and highly conserved sequences are shaded at various degrees.

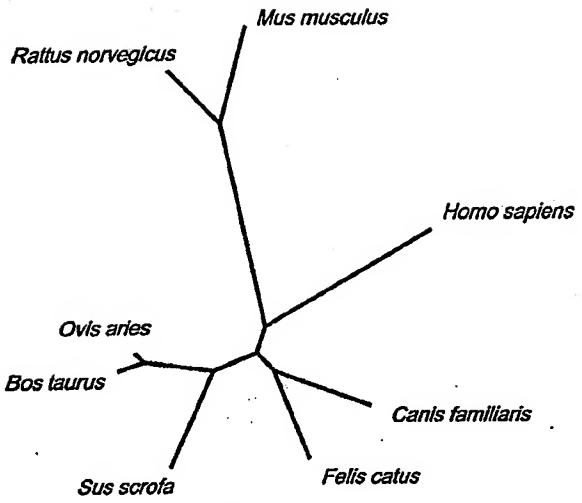

Fig. 4. A phylogenetic consensus tree showing relationships among cat, human, cattle, dog, mouse, rat, sheep and swine based on preproBNP amino acid sequence comparisons.

is consistent with other mammalian species (Tamura et al., 1996). The sequence of the complete mRNA transcript provided in this study indicates that feline BNP has three forms, including a larger precursor of the preproBNP, a proBNP, and a mature BNP. This result is similar to findings in other species (Lewin et al., 1998). The deduced amino acid sequence for feline preproBNP comprised 132-aa residues and the proBNP comprised 106-aa residues. The maximum length of a mature BNP is suggested to be 35-aa residues. Known mammalian precursor convertases cleave at single and/or pairs of basic residues (Docherty and Steiner, 1982; Seidah and Chretien, 1999). The latter is common for most neural and peptide hormones. Seven mammalian precursor convertases identified cleave at the general motif $(K/R) - (X)_n - (K/R)\downarrow$, where $n = 0$, 2, 4 or 6, and X is any amino acid except cysteine (Seidah and Chretien, 1999). Human, dog and pig are reported to have mature BNP forms of 32-aa residues (Seilhamer et al., 1989; Sudoh et al., 1988). Mouse and rat have 45 residues (Kojima et al., 1989; Ogawa et al., 1994). All these mature peptides are consistent with this general cleavage model. Sheep are reported to have two forms of mature BNP, 26- and 29-aa residues in plasma (Aitken et al., 1999). Such isoforms of BNP also fit the cleave sites in this model. Based on this motif, the cat proBNP would have three potential cleave sites that result in mature BNP in the forms of 26-, 29- and 35-aa residues, respectively. Since multiple BNP forms are reported to be present in plasma for other species such as dog, pig and sheep (Aitken et al., 1999; Maekawa et al., 1988), it is possible and likely that cat has multiple forms of mature BNP in vivo. Similarly, cows are likely to have multiple forms of 26-, 29-aa residues of mature BNP although it has not been previously reported. A unique tandem repeat of AUUUA sequence in the 3′ untranslated region was also observed in the feline mRNA. Such repeat units are believed to destabilize BNP mRNA and also exist in other mammalian species (Shaw and Kamen, 1986).

As demonstrated in this study, preproBNP of eight mammalian species showed variations in length and sequence structures. This supports the species-specific actions of BNP across species (Kambayashi et al., 1990). On the other hand, highly conserved sequences were observed in the preproBNP form. For eight mammalian species, 13 amino acid residues were identical in the mature BNP regions. A conserved ring structure of 17 amino acids connected by a disulphide bond is reported in humans (Lewin et al., 1998; Yandle, 1994). Such a ring structure was found to be highly conserved across species. This study showed that ten amino acid residues were identical in this cysteine ring for cat, human, cattle, dog, mouse, rat, sheep and swine. These amino acid residues are also shared in the distinct motif of the 17 amino acid ring structure in ANP and CNP for all known mammalian species. It was found that cat had the same conserved sequences in its disulphide bonded cysteine rings for ANP and CNP (Biondo et al., 2001; Liu et al., 2000). The BNP and ANP genes were found to be located in close proximity on the same chromosome in the human and mouse genomes (Ogawa et al., 1994; Tamura et al., 1996). Sequence similarity and the close location of the two genes suggest a common ancestral origin derived from the more conserved CNP form or gene duplications from the same source (Ohta, 1989; Yandle, 1994). The identical conserved sequence structures of the 17 amino acid ring in the feline BNP, ANP and CNP provide additional evidence in support of this hypothesis. This strongly supports that these cardiac hormones are all members of one family and share related functions in mammalian species.

Based on the similarity of preproBNP sequences, mouse and rat were distinct and formed a closely related group. The human prepropiptide has many unique sequences and appears to have evolved independently from other species. This is supported by previous studies (Kambayashi et al., 1990). Cattle, sheep, and swine shared many common sequences and were clustered together as a closely related group. Such close relationships were in agreement with previous studies (Aitken et al., 1999). As a unique branch, cat and dog were grouped and shared a close relationship. The mature feline BNP had a unique histidine residue at the C-terminal end, shared only with human. It appeared that cat can be an intermediate entry in the phylogenetic relationships among mammalian species. Such a unique relationship suggests that cat could be a model system to study the function of BNP for mammalian species.

Using combined 3′- and 5′-RACE procedures, this study generated a full-length cDNA from feline BNP mRNA. Unlike conventional PCR, the RACE method amplifies nucleic acids using an mRNA template. It is a powerful technique to amplify and characterize unknown sequences without constructing or screening cDNA libraries. With the

availability of cat BNP sequence, antibodies can be generated using synthesized antigenic peptide for immunological assay development. Such assays are efficient and desirable in clinical applications. Further studies using cat BNP will enhance our understanding of BNP functions in cardiac dysfunctions for mammalian species, particularly the cat.

Acknowledgements

The author sincerely thanks Dr J.E. Beever and Dr M. Band for their review and comments to improve the quality of this manuscript. Thanks to Dr H. Remmer for comments on the protein convertase. This study was supported in part by a grant from Bayer AG, Germany (UI99-PRI-S/S-0529 VP022300) to DDS, PFS and ZLL.

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EXHIBIT E

Clinical and Experimental Pharmacology and Physiology (2003) 30, 369–375

HAEMODYNAMIC ACTION OF B-TYPE NATRIURETIC PEPTIDE SUBSTANTIALLY OUTLASTS ITS PLASMA HALF-LIFE IN CONSCIOUS DOGS

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SUMMARY

1. The objective of the present study was to determine the plasma half-life of B-type natriuretic peptide (BNP) in conscious dogs after intravenous administration and to compare this with its haemodynamic effects. In six chronically instrumented dogs, plasma BNP concentrations were measured under basal conditions, during a constant infusion of canine BNP-32 (10 pmol/kg per min; 25 min) to steady state and at nominated time points up to 75 min after stopping the infusion. Concomitant, continuous measurements of mean arterial blood pressure (MAP), heart rate (HR), central venous pressure (CVP) and mesenteric blood flow (MBF) were obtained.

2. Baseline plasma BNP levels were 15.0 ± 2.3 fmol/mL and rose approximately 10-fold to 159 ± 23 fmol/mL after 20–25 min BNP infusion. When the infusion was turned off, plasma BNP levels declined in a biphasic manner, with an initial half-life of 1.57 ± 0.14 min and a terminal half-life of 301 ± 85 min. The metabolic clearance rate of BNP was 2.29 ± 0.34 L/min.

3. The infusion of BNP reduced MAP (approximately 10%), CVP (approximately 65%) and MBF (approximately 25%), whereas haematocrit (approximately 4%) and mesenteric vascular resistance (MVR) increased (approximately 40%; all $P < 0.05$). Plasma BNP levels returned to baseline by 20 min after BNP infusion had been stopped, whereas none of the haemodynamic variables returned to normal by this time. Mean arterial pressure returned to resting levels within 10–15 min after plasma BNP returned to normal. However, CVP, haematocrit and MBF remained substantially below baseline values for more than 20 min after circulating BNP levels had returned to pre-infusion levels. Of these, only mesenteric vascular changes were returned to baseline within 60 min of plasma BNP levels normalizing.

4. These results demonstrate that the removal of BNP from the canine circulation is rapid, similar to observations made regarding the metabolism of circulating atrial natriuretic peptide in dogs. The half-life of BNP in dogs was shorter than that in rats, sheep or humans. However, the haemodynamic actions of BNP substantially outlasted its plasma half-life. Whether this disparity in plasma level and haemodynamic activity of BNP reflects long-lasting activation of second messenger systems or slow recovery from the hydraulic changes at the capillary level, reflected in the haematocrit and CVP, remains to be answered.

Key words: B-type natriuretic peptide, clearance, mesenteric vasoconstriction, metabolism of natriuretic peptides, plasma sequestration.

INTRODUCTION

The cardiac hormone B-type natriuretic peptide (BNP) belongs to a family of natriuretic peptide hormones including atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP). In general, BNP shares many biological actions in common with ANP, including diuretic, natriuretic, hypotensive and smooth muscle relaxant activities.[1] Atrial natriuretic peptide and BNP circulate in the plasma with actions on a number of target organs including the heart, kidney and brain.[2]

Atrial natriuretic peptide metabolism has been studied comprehensively. We know that the metabolic clearance rate of ANP among species is similar, between 2 and 4 L/min.[2] After release into the circulation, most of the ANP is cleared with a very short half-life ranging from 0.5 to 1.0 min in rats, 1–4 min in dogs, 1–3 min in rabbits, approximately 2–3 min in monkeys, approximately 2–5 min in humans (for a review, see Ruskoaho[3]) and approximately 3 min in sheep.[4] The metabolism of BNP is less well studied. Unlike ANP and CNP, which show strong homology across species, BNP differs across species, with only short segments retaining sequence homology.[5] In addition, there are species-specific variations in the structure of the non-guanylyl cyclase-linked natriuretic peptide-C (NP_C) receptor or clearance receptor,[6] which is likely to affect the metabolism of BNP.

There is limited information on the pharmacokinetics of plasma BNP. The metabolic clearance rate of BNP is reportedly between 3 and 6 L/min in humans[7,8] and approximately 8 L/min in sheep.[9] Nishida *et al.*[10] measured BNP clearance of approximately 90 mL/kg per min in dogs with a plasma half-life of approximately 1.5 min. Notably, in that study, animals were anaesthetized. The native, canine form of the peptide was not used and the peptide was injected as a bolus dose rather than as a continuous infusion. Therefore, subsequent plasma measurements may have reflected redistribution, as well as metabolism. In

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Received 1 June 2002; revision 19 December 2002; accepted 22 December 2002.

humans, BNP half-life is approximately 22 min,[8] whereas the half-life of BNP in sheep is approximately 3 min[11] and, in normotensive Wistar-Kyoto rats, half-life is approximately 7 min.[12] Thus, it appears that the plasma half-life and metabolic clearance of BNP are considerably more variable than are those of ANP and, in the dog, half-life and clearance measurements of BNP need to be done with the native peptide, without influence from anaesthesia and from steady state conditions. Therefore, the present study investigated the pharmacokinetics (metabolic clearance rate and plasma half-life) of the species-specific form of infused BNP in normal conscious, greyhound dogs.

We described previously the haemodynamic effects of infused canine-BNP-32 in normal, conscious dogs.[13] Similar to the actions of ANP, infusion of BNP (range 2–20 pmol/kg per min) caused dose-related mesenteric vasoconstriction, diuresis, natriuresis and increases in haematocrit.[13] B-Type natriuretic peptide also lowered arterial pressure and plasma renin activity in these animals. An incidental observation during that study was that some biological actions of BNP appeared to outlast the increase in plasma levels of BNP. This has been further explored in the present study. By measuring serial plasma BNP levels during the decay of exogenous canine BNP from plasma and concomitantly recording haemodynamic changes, we aimed to determine whether there is a significant dissociation between the plasma and biological half-life of BNP in the conscious dog.

METHODS

Preparation of dogs

Experiments were performed on six trained conscious male greyhound dogs (bodyweight 32–36 kg) with chronic indwelling catheters and a mesenteric artery flowprobe. Details of the surgical preparations have been published previously.[13] Briefly, surgical anaesthesia (premedication with acetylpromazine, 2 mg, s.c., and 1.2 mg atropine) was induced with Diprivan (6 mg/kg, propofol; ICI Australia Operations, Melbourne, Victoria, Australia) and maintained by a mixture of halothane and oxygen. Under sterile procedures, via a midline incision, two Barger catheters were inserted approximately 1 cm into the abdominal aorta distal to the renal artery, two vena caval catheters were inserted in the iliolumbar vein and were threaded between 23 and 40 cm downstream toward the heart and a transit-time flowprobe (6 mm ID; Transonic Systems, Ithaca, NY, USA) was placed around the cranial mesenteric artery. The abdominal incision was closed and the catheters and flowprobe lead were tunnelled subcutaneously and exteriorized between the shoulders. A soft canvas coat protected the catheters and probe lead. Oral antibiotics (400 mg metronidazole (Flagyl; Alphapharm, Carole Park, Qld, Australia) and 10 mL septrin (Bactrim; Wellcome Australia, Cabarita, NSW, Australia)) were administered twice daily for 2 days prior to surgery and for 7 days after surgery. Postoperatively, 10 mg Finidyne (flunixin megulamine; Hervot Agvet, Rowville, Victoria, Australia) and 2 mg Promex-2 (acepromazine; Apex Laboratories, St Marys, NSW, Australia) were administered intramuscularly and morphine (10 mg, i.m.; David Bull Laboratories, Mulgrave, Victoria, Australia) was available for analgesia if required.

Dogs were trained prior to surgery and each day during the recovery period to lie quietly on a low padded table in the laboratory. Before the first experiment, the dog was allowed to recover for at least 14 days. During this time, catheters were flushed with saline (0.9% sodium chloride) and their dead spaces were filled with 0.5 mL heparin sodium solution (1000 IU/mL) daily and every alternate day then on.

Experiments

The study protocol was approved by the Alfred Hospital/Baker Medical Research Institute Animal Experimentation Committee and conducted at the Baker Institute. Each experiment consisted of a control period (10 min), a BNP infusion period (25 min) and a recovery period (75 min) to measure the plasma BNP decay curve. Haemodynamic measurements of arterial and venous pressures, as well as mesenteric blood flow (MBF), were collected on-line throughout. Blood samples were taken from an arterial catheter at timed intervals into prechilled tubes containing EDTA for plasma BNP determination (5 mL). One blood sample was taken during the control period to obtain resting levels. Blood samples were also taken after 20 and 25 min of BNP infusion (preliminary experiments indicated that, by these times, the concentration of BNP in the circulation should have reached steady state). Immediately after the 25 min sample was collected, the infusion was turned off. To determine the plasma BNP decay curve, arterial blood samples were taken at fixed intervals of 1, 3, 5, 7, 9, 11, 15, 20, 25, 30, 45, 60 and 75 min after stopping the infusion. Haematocrit (Hct) values were determined from blood samples drawn during the control period, BNP infusion period and the recovery phase (at 15, 30, 45, 60 and 75 min postinfusion).

Peptide

Synthetic canine BNP-32 for infusion (10 pmol/kg per min or approximately 36 ng/kg per min, i.v.; Peninsula Laboratories, Belmont, CA, USA) was prepared in Haemaccel (Behring, Marburg, Germany). Haemaccel is an intravenous solution consisting of 35 g/L degraded gelatin polypeptides cross-linked via urea bridges in water for injection plus physiological concentrations of sodium, potassium, calcium chloride, phosphate and sulphate. The concentration of BNP in the infusate was checked by radioimmunoassay.

Measurements (haemodynamic and hormonal)

Cobe (Lakewood, CO, USA) disposable pressure transducers measured phasic aortic blood pressure and central venous pressure (CVP) from one of the arterial and venous catheters, respectively. Mesenteric blood flow was measured using a Transonic Flowmeter (model no. T208; Transonic Systems, Ithaca, NY, USA) and heart rate (HR) was recorded from the MBF signal using a tachograph (model no. 173; Baker Medical Research Institute, Prahran, Victoria, Australia). Mean arterial pressure (MAP) was derived from a Baker Institute amplifier with a low pass filter set at 0.05 Hz. Mesenteric vascular resistance (MVR) was calculated as (MAP – CVP)/MBF. All data were recorded continuously on an eight-channel Graphtec chart recorder (Linearcorder model no. WR3310; Graphtec, Yokohama, Japan), collected at 300 Hz, digitized via an analogue-to-digital conversion program in 10 s bins and saved by personal computer (model 280; Olivetti, Milan, Italy).

Blood samples were processed by centrifuging the tubes for 10 min at 5420 *g* at 4°C and the plasma was removed and stored at −70°C for radioimmunoassay. Details of the radioimmunoassay for BNP have been published previously.[13] Briefly, BNP was extracted from 1 mL aliquots of plasma using Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) and eluted with 80% methanol in 1% trifluoroacetic acid. Recoveries of added synthetic canine BNP-32 to plasma were approximately 80%.[13] The radioimmunoassay for BNP was performed using commercial porcine BNP-32 antiserum (Phoenix Pharmaceuticals, San Francisco, CA, USA), which cross-reacts 100% with canine BNP-32, [^{125}I]-canine BNP-32 tracer (Phoenix Pharmaceuticals) and the same canine BNP-32 standard that was used in the experiments (Peninsula Laboratories). The interassay coefficient of variation (CV) was 14% (at 30 fmol added/mL plasma) and the intra-assay CV was 11% (at 5 pmol added/mL plasma). All samples from each animal were assayed in the same radioimmunoassay in duplicate to reduce between-measurement variation.

Calculations and statistical analysis

The metabolic clearance rate (MCR) of BNP in individual dogs was calculated as:

$$\text{MCR} = \frac{\text{(infusion rate into plasma)}}{\text{((plateau} - \text{baseline) plasma concentration)}}$$

Plasma BNP levels were adjusted for estimated recovery losses of approximately 20%. B-Type natriuretic peptide decay curves were constructed in individual dogs using a two-compartment model by resolving the plasma BNP concentration versus time curves into two exponential components by the method of residuals.[14] First- (α) and second-order (β) rate constants were determined and the $t_{1/2}$ of BNP was calculated as:

$$t_{1/2} = (\ln 2)/\alpha$$

Statistical analyses on haemodynamic and plasma BNP data were performed using only five time points: control, an average of the two steady state infusion levels and at 10, 20 and 30 min postinfusion. Plasma BNP data were log transformed to equalize variance between steady state and other plasma samples. Haematocrit measurements were not taken from the 20 min blood samples; thus, statistical analyses of Hct were performed on the control, steady state and 10 and 30 min values only. All data were analysed by non-parametric two-way analyses of variance (ANOVA) with the Student–Newman–Keuls' method to adjust for multiple comparisons (Sigmastat, version 2.03; SPSS, Chicago, IL, USA). Statistical significance was taken when $P < 0.05$.

RESULTS

Whole-body clearance and plasma $t_{1/2}$ of BNP

Mean resting plasma BNP concentrations in dogs were 15.0 ± 2.3 fmol/mL. Plasma BNP levels increased approximately 10-fold from resting levels with BNP infusion (Figs 1,2), similar to our previous findings in dogs infused with 10 pmol/kg per min BNP.[13] Plasma BNP levels recorded after 20 and 25 min of BNP infusion were 156 ± 24 and 163 ± 24 fmol/mL, respectively. Because these two BNP levels were not different from each other ($P = 0.77$), the average of these two values in each dog was used for steady state measurements in the pharmacokinetics calculations. The MCR of canine BNP-32 from plasma was 2.294 ± 0.336 L/min. After the infusion of canine BNP-32 was stopped, plasma BNP levels fell rapidly, following first-order kinetics (Figs 1,3), with a calculated slope of the residual (first exponential) phase of the decay curve (α) of 0.458 ± 0.041 fmol/mL per min and a y-intercept at time 0 min of 111 ± 16 fmol/mL (Fig. 3). Plasma BNP levels returned to baseline values by 20 min after the infusion was stopped (Fig. 2). The slope of the terminal (second exponential) phase (β) was 0.004 ± 0.002 fmol/mL per min, with a y-intercept at time 0 min of 20.0 ± 2.3 fmol/mL (Fig. 3). The early return of plasma BNP concentrations towards basal levels followed an elimination $t_{1/2}$ of 1.57 ± 0.14 min.

Haemodynamic responses to BNP

Mean arterial pressure, CVP and MBF fell progressively over the first 15 min of continuous BNP infusion (Fig. 1). Levels of CVP, MBF and MVR were not significantly different between the last two 5 min periods, indicating steady state had been reached. Mean arterial pressure fell from 84.2 ± 3.0 to 80.5 ± 3.1 mmHg ($P < 0.05$) during this period. Heart rate remained unchanged throughout (Fig. 1). By the end of the BNP infusion period, compared with basal levels, MAP had fallen by 11 ± 3 mmHg (approximately 12%; $P = 0.011$), CVP had fallen by 1.0 ± 0.3 mmHg (approximately 67%; $P < 0.001$) and MBF had fallen by 85 ± 14 mL/min (approximately 25%; $P < 0.001$; Fig. 2).

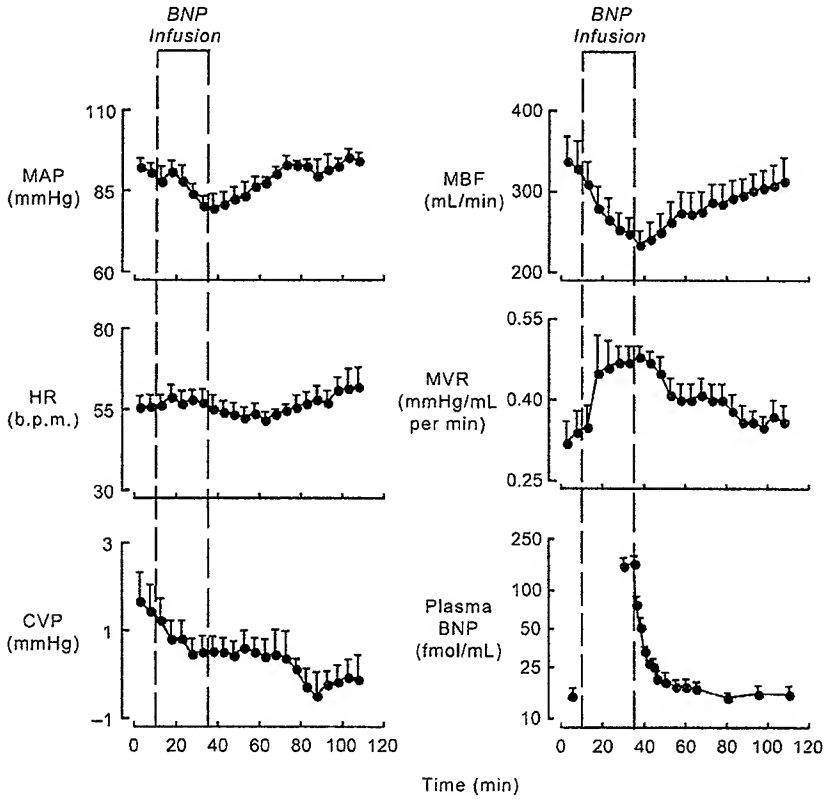

Fig. 1 Profile of haemodynamic measurements and plasma B-type natriuretic peptide (BNP) levels in conscious dogs ($n = 6$) before (0–10 min), during (10–35 min) and after (35–110 min) synthetic canine BNP-32 infusion at 10 pmol/kg per min, i.v. Results are expressed as the mean±SEM, between animals. The area between the dashed lines represents the BNP infusion period. MAP, mean arterial pressure; HR, heart rate; CVP, central venous pressure; MBF, mesenteric blood flow; MVR, mesenteric vascular resistance.

With the fall in MBF, there was a corresponding increase in MVR of 0.137 ± 0.020 mmHg/mL per min (approximately 40%; $P < 0.001$; Fig. 2). Haematocrit increased during BNP infusion by 1.7 ± 0.5% red blood cells (approximately 4%; $P < 0.017$) from resting levels (Table 1).

Once the BNP infusion was turned off, haemodynamic recovery was much slower than the onset (Figs 1–3). Only MAP returned to baseline levels within 40 min of recovery (Figs 1,2). In contrast, mesenteric vascular changes took approximately 60 min to recover to baseline levels (Figs 1–3). The dissociation between plasma BNP levels and MVR over the first 30 min is further illustrated in Fig. 3. During this time, the BNP-induced increase in MVR (to 46 ± 10% of pre-infusion levels) slowly returned towards pre-infusion levels, with a rate that more closely resembled the terminal phase of the BNP decay curve than the initial exponential phase (Fig. 3). Changes in CVP (Fig. 2) and Hct (Table 1) were even more long lasting and, up to 75 min after the BNP infusion was turned off, CVP was still below (Fig. 2) and Hct above baseline levels (Fig. 1).

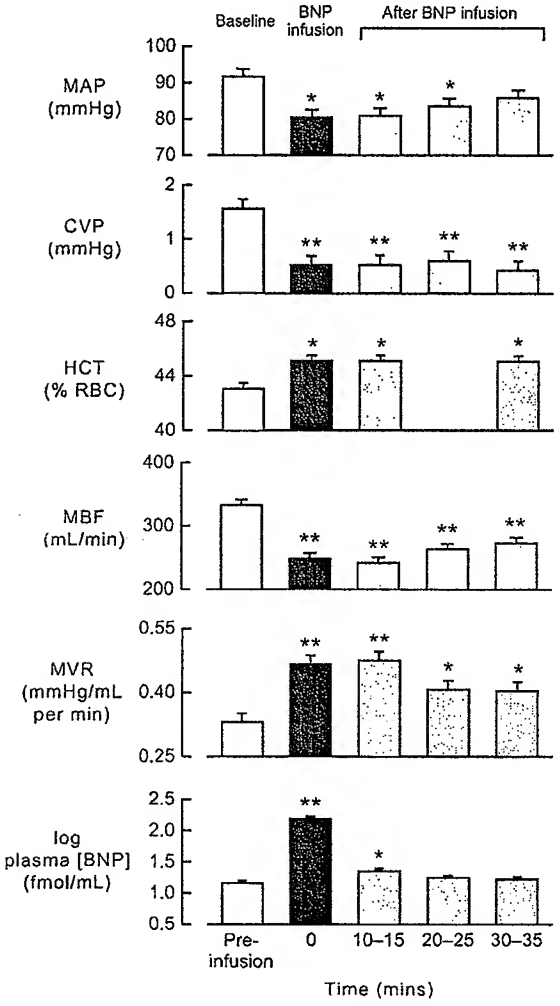

Fig. 2 Prolonged haemodynamic effects of canine B-type natriuretic peptide (BNP)-32 infusion, lasting longer than the peptide in plasma, in six dogs. For haemodynamic measurements: (□), 10 min averages of the pre-infusion time; (■), 5 min average of the last 20–25 min of the BNP infusion; (▩), 5 min averages at 10–15 min, 20–25 min and 30–35 min after BNP infusion was stopped. The plasma BNP level during infusion (■) was the average of two separate measurements at 20 and 25 min. Plasma BNP levels for the 10–15 min period were averaged from 9 and 11 min samples. There was no haematocrit (HCT) measurement between 15 and 30 min. Results are expressed as the mean±SE of the least square mean from within animal variance (from two-way ANOVA). The dotted line represents baseline levels. $*P < 0.05$, $**P < 0.001$ compared with the pre-infusion level. MAP, mean arterial pressure; CVP, central venous pressure; MBF, mesenteric blood flow; MVR, mesenteric vascular resistance; RBC, red blood cells.

DISCUSSION

The present study reports the pharmacokinetics and time-related haemodynamic responses during infusion of species-specific BNP and recovery after infusion ceased in normal, conscious dogs. Our findings indicate that metabolism (clearance from plasma and disappearance rate) of BNP in these animals is substantially faster than its biological effects following exogenous infusion.

Canine BNP half-life and MCR: Comparison with other species

These experiments provide new pharmacokinetic data for BNP in the conscious dog. The MCR of canine BNP-32 in conscious dogs was approximately 2.3 L/min. Previously, Nishida *et al.*[10] had reported that BNP was cleared from the circulation of dogs at

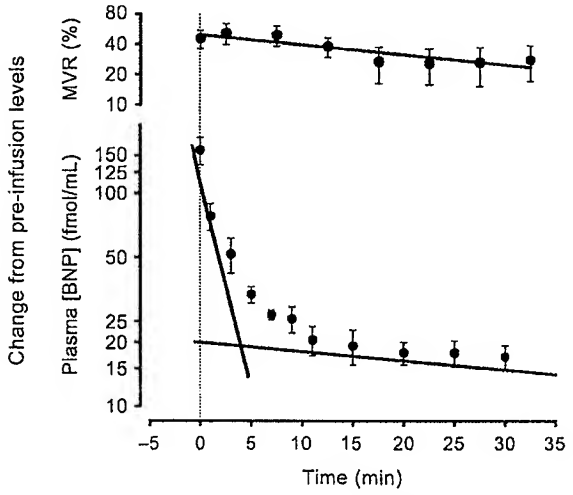

Fig. 3 Expanded scale of the 30 min period immediately after infusion of B-type natriuretic peptide (BNP) was stopped, showing the percentage change from pre-infusion levels of mesenteric vascular resistance (MVR) and the change in plasma BNP concentration. In both cases, the y-axis is a log scale. Data are the mean±SEM (between animal variation). The line on the MVR graph represents the linear regression equation of $y = 49.5\% - 0.49x$ with $r^2 = 0.781$ ($P < 0.05$). Lines on the plasma BNP graph represent the two mean linear regressions ($n = 6$ dogs) fitted using a two-compartment model by the method of residuals[14] to resolve the exponential decay relationship between plasma BNP concentration versus time (see Results in text).

a rate of 90 mL/kg per min, which is equivalent to approximately 2.7 L/min, if adjusted for a bodyweight of 30 kg, similar to our dogs. There are several differences in the experimental design of the study of Nishida *et al.*[10] compared with the present study. Nishida *et al.*[10] used a non-species-specific form of BNP, a single bolus injection of the peptide was used rather than an infusion to steady state, requiring a different method of pharmacokinetic analysis of the data (one-compartment model), and metabolism and clearance of BNP may have been influenced by anaesthesia.[10] Nevertheless, results from the two studies are remarkably similar. The metabolic clearance rate of BNP has been reported in two other species. Our results in the dog are at the lower end of the range reported for humans, of approximately 3–6 L/min,[7,8] and less than 50% of the MCR in sheep, approximately 8 L/min.[9] The method we used to measure MCR is based on the assumption that baseline levels of BNP remain constant during infusion of BNP to steady state. Although this assumption may not have held with the changing haemodynamics, any overestimate of MCR with this method is likely to be in the order of 5% because baseline levels of BNP were approximately 10% of those during steady state infusion.

Plasma half-life of canine BNP in the conscious dog was approximately 1.5 min. Nishida *et al.*[10] previously reported that the half-life of bolus-injected non-homologous BNP was approximately 1.5 min in the anaesthetized dog. In humans, BNP half-life is approximately 22 min,[8] whereas the half-life of BNP in sheep is approximately 3 min[11] and, in normotensive Wistar-Kyoto rats, the half-life of BNP is approximately 7 min.[12] Given these differing removal rates of BNP from plasma across species, it is important when trying to experimentally mimic the physiological or pathophysiological condition *in vivo* to tailor the rate of infusion to the species being studied.

How do the pharmacokinetics of BNP and ANP compare in the dog?

Elimination of BNP from the canine circulation occurred in a biphasic manner. Most of the peptide was eliminated within a short time, with BNP in the plasma falling rapidly and exponentially during the first 5 min after ceasing intravenous infusion. Thereafter, plasma BNP levels declined more slowly to reach basal values approximately 20 min after the infusion had been discontinued. This is similar to the profile of response we reported previously for plasma ANP in conscious dogs,[15] consistent with the short half-lives of both these natriuretic peptides in plasma (approximately 1.5 min for BNP and approximately 1 min for ANP[15]). The metabolic clearance rate of ANP is high in dogs,[10,15,16] approaching their cardiac output (approximately 2–3 L/min[17,18]). The metabolic clearance rate of BNP (approximately 2–3 L/min) indicates that most of the circulating BNP in dogs is also cleared in one circulation through peripheral tissues. Indeed, BNP appears to be metabolized rapidly at rates similar to other vasoactive hormones, such as angiotensin II and vasopressin.[19,20]

Haemodynamic effects of BNP in dogs

The much longer-lasting haemodynamic effects of BNP compared with plasma levels are intriguing. We have described previously the mesenteric vasoconstrictor actions of BNP, which are very similar to ANP, in conscious dogs.[13] Although we know these vascular effects are not the result of reflex sympathetic activation,[13,17,21] the mechanism of action of these hormones to constrict mesenteric vasculature is uncertain. We have shown previously that a secondary factor from the gut may be responsible for ANP-induced mesenteric vasoconstriction[22] and that this may require the activation of α-adrenoceptors (CJ Thomas and RL Woods, unpubl. obs., 1995–97). Given the common gut vascular target and similar potency of ANP and BNP,[13] it seems reasonable to assume they share the same mechanism of action. Thus, a secondary factor with vasoconstrictor properties that is active for longer than the plasma half-life of BNP could be responsible for the uncoupling of plasma BNP levels and mesenteric vasoconstriction.

The other major haemodynamic changes that outlasted circulating levels of BNP were reductions in CVP and increases in Hct. Although the study is inherently limited through the absence of time-matched control data, evidence from previous experiments using an identical protocol suggests the long-term shift in haemodynamics truly reflects the effects of BNP. Under conditions identical to those of the present study, CVP fell by < 0.5 mmHg over the first 2 h and no other cardiovascular variable changed during more than 3 h of continuous monitoring.[13] It is widely understood that ANP, and likely BNP, acts on capillary permeability to shift plasma out of the vascular compartment. In the present study, such a permeability shift may be reflected in the increased Hct and reduced venous pressure. The much slower passive return of sequestered plasma back into the vasculature, compared with the more active hormone-mediated process driving it out, is presumably the main reason for the delay in return of CVP and Hct. In addition to reduced venous pressures, consequences of the volume shift are reduced venous return and cardiac filling pressures resulting in lowered cardiac output. This is the primary cause of the modest hypotension,[9,13,23] although with BNP there may also be reductions in non-splanchnic vascular resistance (RL Woods, unpubl. obs. in sheep, 2001–2) in addition to the falls in cardiac output that contribute to the lowered blood pressure. In the present study, arterial pressure returned to baseline long before the venous pressures recovered. The mechanism for this is unknown,

Table 1 Haematocrit levels before (pre-infusion control), during and at timed intervals (indicated by min) after B-type natriuretic peptide infusion (recovery)

	Pre-infusion control	During BNP infusion	Recovery 15 min	30 min	45 min	60 min	75 min
Hct (% red blood cells)	43.1 ±2.1	44.7 ±0.3*	45.1 ±0.3*	45.1 ±0.3*	44.7 ±0.3*	44.5 ±0.3*	44.9 ±0.3*

Data are the mean±SEM (to indicate between-animal variation) for pre-infusion control and are mean±SE of least square mean (to indicate within-animal variation from ANOVA) for the remaining values. Blood was collected at 20 and 25 min into the infusion and the average haematocrit (Hct) value taken as the steady state level during infusion. Otherwise, results are from blood collected at the designated time points. *$P < 0.05$ compared with the pre-infusion level (two-way ANOVA with Bonferroni correction for multiple comparisons).

but a possible explanation is passive recoil of vasculature around a contracted blood volume, contributing to an increased total peripheral resistance. In addition, we have shown that BNP in humans is sympathoinhibitory[24] and the lack of reflex activation on capillary flux may contribute to the delayed return of plasma after BNP.

Natriuretic peptide-induced vasoconstriction: Role of degradation mechanisms?

The three natriuretic peptides are inactivated and removed from the circulation by two major metabolic clearance pathways: (i) receptor-mediated internalization; and (ii) enzymatic digestion.[25] The first pathway is mediated by NP_C (or so-called clearance) receptors and the second is mediated primarily by neutral endopeptidase (NEP).[25] Excretion of the natriuretic peptides in urine also occurs, but, as we have demonstrated in dogs for ANP,[15] contributes minimally to whole-body clearance of that peptide. Among the three natriuretic peptides, BNP binds to the NP_C or clearance receptor with the lowest affinity (ANP > CNP > BNP) in most species[26] and, in humans, BNP is less susceptible to degradation by NEP, resulting in a longer half-life.[27] Plasma concentrations of ANP and BNP are augmented following vasopeptidase inhibition (which coinhibits NEP and angiotensin-converting enzyme (ACE) in dogs with mild heart failure).[28] Brandt *et al.*[18] found infusion of C-ANP-(4–23), a specific ligand for the NP_C receptor, reduced the metabolic clearance rate of ANP in conscious dogs, implicating the NP_C receptor in the degradation of ANP in the dog. From the results of present and previous[15] studies, it seems likely that, in the dog, clearance pathways for ANP and BNP will be similar because the half-lives and MCR are so close. That is, both NEP and NP_C receptor probably contribute to the plasma clearance of BNP in the dog.

Conclusions

The current studies extend our understanding of BNP metabolism in normal, conscious dogs. We found that the pattern of BNP disappearance in canine plasma closely resembles that of ANP. In addition, the present study highlighted that the haemodynamic actions of BNP substantially outlast its plasma half-life.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia and the Ramaciotti Foundation. We appreciate the assistance of Simon Fitzpatrick (Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne) in completing these experiments.

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EXHIBIT F

J Vet Intern Med 2003;17:172–177

Brain Natriuretic Peptide Concentration in Dogs with Heart Disease and Congestive Heart Failure

Kristin A. MacDonald, Mark D. Kittleson, Coralie Munro, and Philip Kass

Plasma brain natriuretic peptide concentration ([BNP]) is high in humans with cardiac disease and is further increased with congestive heart failure (CHF). The hypotheses of this study were that dogs with moderate to severe mitral regurgitation due to myxomatous mitral valve disease (MVD) would have increased plasma [BNP] compared to normal dogs, that plasma [BNP] would be higher in dogs with CHF, and that plasma [BNP] would predict premature death from cardiovascular disease. The study population consisted of 34 dogs: 9 normal dogs and 25 dogs with MVD. Patients were divided into 4 groups: group I—10 dogs with moderate to severe MVD and no radiographic evidence of CHF; group II—6 dogs with severe MVD and mild CHF; group III—7 dogs with severe MVD and moderate CHF; and group IV—2 dogs with severe MVD and severe CHF. Diagnostic tests included thoracic radiographs, an echocardiogram, a serum chemistry profile, and the measurement of plasma [BNP] by a canine-specific radioimmunoassay. There was a significant positive correlation between the plasma [BNP] and heart disease/failure groups ($P = .0036$). Plasma [BNP] increased with progressively increasing severity of MVD and CHF. Group I dogs had higher plasma [BNP] than did control dogs ($P < .0001$), and plasma [BNP] was higher in dogs with CHF (groups II–IV versus group I; $P = .012$). Plasma [BNP] was also weakly positively correlated with left atrial size ($r = 0.43$, $P = .04$). For every 10-pg/mL increase in plasma [BNP], the mortality rate over 4 months' time increased approximately 44%.

Key words: Diagnostic test; Mortality; Myxomatous mitral valve disease.

Plasma brain natriuretic peptide concentration ([BNP]) is high in human patients with cardiac disease, is higher in patients with congestive heart failure (CHF), and is a good prognostic indicator of premature death from cardiovascular disease.[1–10] There is minimal information regarding plasma [BNP] in normal dogs or in dogs with heart disease.[11–13]

BNP is a hormone that is synthesized in the cardiac atria and ventricles.[14] The induction of BNP synthesis is mostly due to increased ventricular wall stress, but local and circulating endothelin-1 (ET-1) inductions also increase BNP synthesis. Both increased systolic and increased diastolic wall stresses induce BNP synthesis.[15,16] BNP's biologic effects include primary natriuresis and the inhibition of renin and ET-1 release, resulting in secondary vasodilation and natriuresis.[17]

Plasma [BNP] is high in many different cardiac diseases in several species.[6,11,12,18–20] Plasma [BNP] is high in humans with dilated cardiomyopathy as well as in dogs and pigs with pacing-induced myocardial failure.[6,9,11,21] Plasma [BNP] is also high with naturally occurring myxomatous mitral valve disease (MVD) in humans and dogs as well as with experimentally created mitral regurgitation in dogs.[12,13,22] Diastolic dysfunction, hypertrophic cardiomyopathy, and systemic hypertension are other diseases associated with high plasma [BNP] in humans.[2,3,20,23–26] Because of its accuracy, plasma [BNP] is rapidly becoming a standard test for detecting the presence of left ventricular dysfunction and CHF in human medicine.[4,5,27,28] The measurement of plasma [BNP] is also becoming an important prognostic indicator of cardiovascular mortality in humans.[9]

The hypotheses of this study were that dogs with moderate to severe MVD would have increased plasma [BNP] compared to normal dogs and that plasma [BNP] would increase further when CHF is present. We also hypothesized that plasma [BNP] would predict early cardiovascular mortality.

Materials and Methods

The study population consisted of canine patients with mitral regurgitation due to MVD that were presented to the University of California at Davis Veterinary Medical Teaching Hospital (VMTH) Cardiology Service from August 2000 to November 2000 and normal dogs owned by students, faculty, and staff of the VMTH. A physical examination and an echocardiogram were performed on each normal dog to rule out the presence of cardiac disease. Clients signed a consent form for the inclusion of their dog in the study. Cardiac patients were placed in 1 of 4 groups on the basis of radiographic and echocardiographic assessment of disease severity and radiographic assessment of the presence and severity of CHF at the time of the study. The investigators blindly placed the dogs in the heart disease groups before obtaining the plasma [BNP] results. CHF was diagnosed on the basis of radiographic evidence of caudodorsally distributed interstitial to alveolar pulmonary infiltrates and left atrial enlargement. Mild CHF was defined as radiographic evidence of mild perihilar to caudodorsal interstitial pulmonary infiltrates.[29] Moderate CHF was characterized by a moderate density of caudodorsal interstitial pulmonary infiltrates on thoracic radiographs.[29] Severe CHF was defined as radiographic evidence of alveolar pulmonary infiltrates.[29] Dogs with moderate to severe mitral regurgitation and no CHF were placed in group I, dogs with severe mitral regurgitation and mild CHF were placed in group II, dogs with severe mitral regurgitation and moderate CHF were placed in group III, and dogs with severe mitral regurgitation and severe CHF comprised group IV. Many of the patients in group I had been in CHF previously and were on medications for CHF at the time of the study. Patients were excluded from the study if there was evidence of clinically relevant systemic disease other than heart failure, as determined by physical examination, CBC, serum chemistries, urinalysis, and thoracic radiographs. Because renal failure causes increas-

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Submitted May 10, 2002; Revised August 7, 2002; Accepted October 23, 2002.

0891-6640/03/1702-0008/$3.00/0

Table 1. Current heart failure medication administered to patients in each heart disease group.

Medication	Group I (n = 10)	Group II (n = 6)	Group III (n = 7)	Group IV (n = 2)
Furosemide	2	0	0	1
ACE inhibitor	0	0	1	0
Digoxin	0	0	0	0
Spironolactone	0	0	0	0
Hydrocodone	1	0	0	0
Combination treatment:				
Lasix plus ACE inhibitor	1	1	3	1
Lasix, ACE inhibitor, digoxin		2	1	
Lasix, ACE inhibitor, hydrocodone	1		1	
Lasix, ACE inhibitor, digoxin, spironolactone			1	
% Dogs in each group on medication	50%	50%	100%	100%

ACE, angiotensin-converting enzyme.

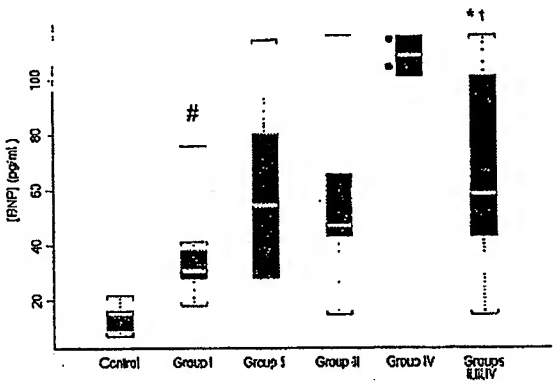

Fig 1. Distribution of plasma brain natriuretic peptide concentration ([BNP]) in control dogs and dogs with heart disease. Boxes show (from bottom to top) the 25th, 50th, and 75th percentiles. Whiskers extending from boxes capture approximately 95% of the data. Extreme observations are represented by lines. # Significant difference between group I and control; $P = .0001$. * Significant difference between groups II–IV and group I; $P = .012$. † Significant difference between groups II–IV and control; $P < .0001$.

es in plasma [BNP], dogs with a serum urea nitrogen concentration >50 mg/dL, a serum creatinine concentration >3 mg/dL, or both were excluded from the study.

Echocardiography was performed with an Agilent Sonos 5500 ultrasound machine.[a] Measurements and calculations included left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), shortening fraction, left atrial : aortic diameter ratio obtained from 2-dimensional echocardiography in the right parasternal short-axis view (LA : Ao), color flow Doppler echocardiography, and continuous wave Doppler echocardiography–derived mitral regurgitant velocity. LVEDD was indexed (LVEDDI) to 1.44 $kg^{0.32}$, and LVESD was indexed (LVESDI) to 0.69 $kg^{0.41}$.[29,30] Radiographic heart size was semiquantified by calculating a vertebral heart scale from the lateral view of thoracic radiographs.[31] Plasma [BNP] was measured with a commercial, competitive radioimmunoassay kit specific for canine BNP-32.[b] All materials and buffers were supplied with the kit and used in strict accordance with the kit guidelines. Radioimmunoassay measurement of plasma canine BNP-32 has been validated in a previous study.[13] Blood samples were collected in polypropylene tubes containing EDTA and aprotinin. Samples were immediately centrifuged at 0°C, plasma was frozen at −70°C, and samples were batched for analysis. All plasma samples were extracted before the assay according to the manufacturers' instructions.

Owners of the dogs were verbally contacted to determine short-term patient survival data at 4 months after the initial diagnosis and long-term patient survival data at 18 months after the initial diagnosis. Deaths were classified as cardiac or noncardiac in origin.

Statistical analyses were performed by Statview 5[c] and StatXact.[d] The Jonckheere-Terpestra test was used to compare the trend in BNP concentration across the 4 groups of dogs with MVD and progressively increasing severity of CHF. The Mann-Whitney *U*-test was used to compare the plasma [BNP] of the control group with group I heart disease as well as to compare the plasma [BNP] of groups II–IV (dogs with severe MVD and CHF) with the control group and group I. Simple linear regression was used to evaluate the following independent variables with regard to the dependent variable of plasma [BNP]: LVEDDI, LVESDI, LA : Ao, and vertebral heart score. Pearson's product-moment correlation coefficients were calculated. Statistical significance was defined as $P \leq .05$. A Cox proportional hazards regression model was used to evaluate the relationship between plasma [BNP] at the time of diagnosis as well as 4 and 18 months after the diagnosis. The proportionality and linearity assumptions of the model were assessed by likelihood ratio tests. Results of this model are presented as mortality rate ratios (MRRs) and 95% confidence intervals (95% CI).

Results

There were 25 dogs with MVD in the study and 9 normal control dogs. Ages of control dogs ranged from 4 to 10 years (mean, 6.9 years), and weights ranged from 7 to 55 kg (mean, 31.3 kg). Patient ages ranged from 18 months to 14 years (mean, 10.3 years), and weights ranged from 3.5 to 38 kg (mean, 16.6 kg). There were 10 dogs in group I, 6 dogs in group II, 7 dogs in group III, and 2 dogs in group IV. Table 1 depicts the current medications administered to patients in each heart disease group.

There was a significant ordinal correlation between the plasma [BNP] and heart disease groups ($P = .0036$) (Fig 1). Plasma [BNP] was significantly high in the patients with MVD and no CHF (group I) when compared to the control dogs ($P < .0001$). Additionally, plasma [BNP] was greater in dogs with MVD and CHF (groups II–IV) than in control dogs ($P < .0001$) as well as in dogs with MVD only (group I) ($P = .012$) (Fig 2).

There was minimal overlap in the plasma [BNP] of normal dogs and group I patients. A plasma [BNP] cutoff of 23 pg/mL provided the best sensitivity (86%; 95% CI, 65–97%) and specificity (100%; 95% CI, 72–100%). Similarly, a plasma [BNP] cutoff of 35 pg/mL provided the best sensitivity (86%; 95% CI, 57–98%) and specificity (70%; 95% CI, 35–93%) for distinguishing between dogs with heart failure due to MVD and dogs with MVD but without heart failure.

Four-month survival data were available for 21 of the 25 dogs. One dog was censored from the short-term survival analysis because it had undergone surgical repair of the mitral valve. Nine of the 20 remaining dogs with MVD died or were euthanized because of worsening CHF early, within

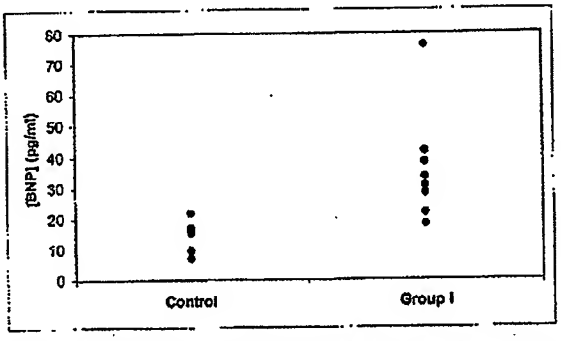

Fig 2. Scattergram of plasma brain natriuretic peptide concentration ([BNP]) in control dogs and group I heart disease dogs with moderate to severe mitral valve disease (MVD) but no heart failure.

4 months of the initial evaluation. A dose-response relationship between plasma [BNP] and the rate of death within 4 months of diagnosis (ie, short-term survival) was found: for every 10-pg/mL increase in plasma [BNP], the mortality rate increased approximately 44% (MRR = 1.4; 95% CI, 1.1–1.9). For example, a dog with a plasma [BNP] of 100 pg/mL had a 12.6-fold greater rate of mortality over 4 months' time than a dog with a plasma [BNP] of 30 pg/mL (MRR = 12.6; 95% CI, 6.8–82.3). Only 1 dog died of a noncardiac cause at 6 months after the initial diagnosis and was eliminated from the long-term survival analysis. Fifteen of the remaining 19 dogs (79%) died or were euthanized because of worsening CHF by 18 months after initial diagnosis. There was no relationship between plasma [BNP] and long-term patient survival at 18 months after the initial diagnosis.

Simple linear regression showed a statistically significant but clinically weak correlation between plasma [BNP] and LA : Ao ($r = 0.43$; $P = .04$). There was no significant correlation between [BNP] and the other measured variables.

Discussion

This study showed that plasma [BNP] was high in dogs with moderate to severe MVD and was further increased in dogs with CHF due to severe MVD. There are only 2 published veterinary studies that have evaluated plasma [BNP] in dogs with naturally occurring cardiac disease. One study measured plasma [BNP] in 19 dogs with CHF and MVD (n = 17) or dilated cardiomyopathy (n = 2).[13] Another study measured plasma [BNP] in 76 Cavalier King Charles Spaniels with MVD of varying severity.[12] Both studies found that plasma [BNP] was high only in dogs with moderate and severe CHF. In contrast, the present study found that plasma [BNP] was high in dogs with moderate to severe myxomatous MVD in the absence of CHF and that plasma [BNP] was further increased with CHF due to severe MVD. Plasma [BNP] increased as CHF progressively worsened.

Similar to our study, a study in humans found that plasma [BNP] was high with moderate to severe MVD and that plasma [BNP] increased with worsening signs and heart failure class.[22] That study reported that a plasma [BNP] >36 pg/mL was predictive of patients who later decompensated and required surgery for valve replacement. In the current study, a plasma [BNP] >35 pg/mL was moderately sensitive and specific for differentiating dogs that were not in heart failure from dogs with CHF.

The present study showed that, over a 4-month evaluation period, as plasma [BNP] at the time of diagnosis increased, the rate of death increased. There are no other veterinary studies that have evaluated plasma [BNP] as a prognostic indicator of death from cardiovascular disease. However, plasma [BNP] is a good prognostic indicator of cardiovascular mortality in humans with CHF due to dilated cardiomyopathy as well as ischemic heart disease.[9,10,32] One study measured plasma [BNP], plasma atrial natriuretic peptide concentration, and plasma norepinephrine concentration in persons with CHF due to dilated cardiomyopathy or ischemic heart disease. Only plasma [BNP] was an independent predictor of risk of death.[9] Plasma [BNP] also provided prognostic information independent of other hemodynamic variables such as left ventricular ejection fraction.[9] Another study surveyed 1,640 people randomly chosen from the general population and found that plasma [BNP] independently predicted death due to left ventricular dysfunction.[33]

The measurement of plasma [BNP] has been used in human medicine as a screening test to detect asymptomatic left ventricular systolic dysfunction. One study evaluated 1,252 randomly selected people and found that a plasma [BNP] >18 pg/mL was moderately sensitive (77%) and specific (87%) for detecting asymptomatic left ventricular systolic dysfunction.[27] This value is very similar to the value of 23 pg/mL found in the current study for detecting heart disease. Another study measured plasma [BNP] in 200 patients referred for echocardiography and found that a high plasma [BNP] was highly specific (98%) for detecting left ventricular systolic dysfunction.[28]

Increased diastolic wall stress also causes increased synthesis and release of BNP from ventricular myocardium.[15] Plasma [BNP] is high in humans with diastolic dysfunction.[20,23] The measurement of plasma [BNP] has been used as a screening test for the presence of asymptomatic diastolic dysfunction and left ventricular hypertrophy.[24] A plasma [BNP] >25 pg/mL was useful for detecting left ventricular diastolic dysfunction and hypertrophy in that study. Again, this value is very similar to the value of 23 pg/mL found in the current study for detecting heart disease.

Recently, a rapid bedside plasma [BNP] test became available for humans, and it has been used in the emergency room setting as a screening tool for detecting CHF.[6] Two studies have evaluated plasma [BNP] in dyspneic patients who were presented to urgent care hospitals.[4,5] Both studies found that plasma [BNP] was higher in patients with CHF than in patients without CHF. In one of the studies, 30 of 250 dyspneic patients were misdiagnosed clinically. When a plasma [BNP] measurement was used, there was only 1 misdiagnosis.[5] In addition, the measurement of plasma [BNP] was highly specific for detecting the presence or absence of CHF, and the diagnostic accuracy was not improved by obtaining thoracic radiographs.[5] To our knowledge, no veterinary studies have measured plasma [BNP] as a screening test for CHF. A future study should evaluate

the measurement of plasma [BNP] as a test to discriminate between primary respiratory disease and CHF in dogs presented for dyspnea.

Plasma [BNP] has also been used to predict a human patient's response to treatment.[32] In one study, serial plasma [BNP] measurements of hospitalized CHF patients predicted treatment outcome and mortality. Patients whose plasma [BNP] declined during hospitalization were much less likely to be rehospitalized or die than patients whose plasma [BNP] increased during hospitalization. Future veterinary studies should evaluate plasma [BNP] during the treatment of CHF and assess whether the measurement of plasma [BNP] can be used to help tailor the medical treatment of CHF.

Variables such as age, posture, and time of day could theoretically change plasma [BNP]. The effect of aging on plasma [BNP] has been studied in humans and dogs. Although plasma [BNP] has been shown to mildly increase with age in humans, there is no correlation between plasma [BNP] and age in dogs.[24,34] BNP is not affected by postural changes, and there is no circadian rhythm of secretion.[35,36] Acute (ie, minutes) volume overload does not affect plasma [BNP].[37] However, within 1 hour of producing an increase in ventricular wall stretch, BNP gene expression is activated in the left ventricle.[19]

The primary direct biologic effect of BNP is the production of natriuresis in the proximal and distal renal tubules.[38] BNP also inhibits endothelin-1 and renin secretion and therefore causes secondary decreases in angiotensin II (AT-II) and aldosterone concentrations.[39–41] Consequently, it produces secondary biologic effects that include vasodilation and natriuresis.[42] Natriuretic peptides also possess antiproliferative properties in the mesangial cells of the kidney, astrocytes, endothelial cells, cardiac fibroblasts, vascular smooth muscle cells, and cardiomyocytes.[43–45] BNP inhibits collagen synthesis, possibly by ET-1 inhibition.[46–48] BNP is degraded by neutral endopeptidases in the myocardium, lungs, and kidneys.[49]

High plasma [BNP] in human patients with moderate to severe cardiac disease is likely due to increased left ventricular synthesis.[8,11] However, BNP synthesis and secretion are different, depending on the presence or absence of cardiac disease. BNP is synthesized as a prohormone, and most of the active hormone is released into circulation without being stored in granules.[50,51] In the absence of cardiac disease, the tissue concentration of BNP is greatest in the atria.[14] Given the large mass of the ventricles, the total cardiac content of BNP is greater in the ventricles. In fact, 50–60% of circulating BNP is synthesized in the ventricles in normal individuals.[14] There appears to be a different regulation of atrial and ventricular BNP synthesis.[11,14,52,53] BNP is secreted from ventricular myocytes quickly after synthesis via a constitutive pathway, but in the atria, it is stored in granules and released by a regulatory pathway.[17,38,50,51,54] ET-1 causes ventricular but not atrial synthesis of BNP in deoxycorticosterone acetate–salt hypertensive rats.[55] During early left ventricular dysfunction, plasma [BNP] is increased, and atrial tissue contents of BNP and BNP messenger RNA (mRNA) are also increased.[11] Once CHF is present, plasma [BNP] and left ventricular BNP mRNA are further increased.[11] The activation of BNP transcription is one of the earliest and most reliable markers of ventricular cardiomyocyte hypertrophy.[56] Ventricular BNP secretion also increases with increasing severity of left ventricular dysfunction.[8]

The major determinant of BNP synthesis and secretion is wall stress.[14] Both mechanical stretch and pressure overload induce BNP gene expression and increase BNP secretion.[19,57] However, BNP synthesis can be induced by both load-dependent and load-independent mechanisms.[52] There is debate regarding whether wall stress directly causes BNP synthesis and release or whether increased wall stress causes local autocrine or paracrine factors to induce BNP synthesis. Possible local factors may include the renin-angiotensin system and ET-1. ET-1 is synthesized and secreted from endothelial cells and cardiomyocytes and is released during endothelial stretch or pressure overload.[58] ET-1 has been shown to induce ventricular BNP synthesis and cause an increase in plasma [BNP].[52,53] Although cardiomyocyte stretch causes acute AT-II release, one study showed that AT-II is not required for stretch to trigger increased BNP gene expression in the atria and ventricles.[59] Experiments with cultured neonatal cardiomyocytes have shown that mechanical stretch in the absence of neurohumoral control increases gene expression and secretion of BNP.[57] The same experiment showed that an AT-II receptor blocker, an ET-1 receptor blocker, or the combination of both AT-II and ET-1 receptor blockers reduce mechanical strain–induced BNP gene transcription by 50%.[60] Therefore, both mechanical strain and neurohumoral stimulation appear to be important inducers of BNP production. In humans with dilated cardiomyopathy, BNP-expressing cardiomyocytes have been found in the subendocardial layer, fibrous areas, and perivascular regions.[61] These findings show that, in addition to global wall stress, there may be regional factors that induce BNP synthesis in the left ventricle.

Although plasma [BNP] was increased in the patients of our study with MVD and CHF, plasma [BNP] was not markedly related to radiographic heart size or echocardiographic-derived chamber sizes, including LVEDDI and LVESDI. By simple linear regression, there was a very weak relationship between left atrial size and plasma [BNP]. There is minimal clinical relevance with such a low correlation coefficient. The echocardiographic variables of left ventricular size are not good approximations of left ventricular wall stress. Because wall stress is the major stimulus for the synthesis and release of BNP, the calculation of ventricular wall stress would have been more appropriate than the measurement of left ventricular echocardiographic dimensions. However, left ventricular wall stress is difficult to calculate and requires invasive procedures.

The major limitation of this study is the small number of control dogs and patients included. Although there were statistically significant results, further studies should be carried out in larger populations of dogs to verify and refine these findings. Other potential areas of BNP research include the measurement of plasma [BNP] as a screening test for differentiating dyspneic patients with CHF from patients with primary respiratory disease, the measurement of plasma [BNP] in other cardiac diseases such as hypertrophic cardiomyopathy and dilated cardiomyopathy, and the measurement of plasma [BNP] for the therapeutic monitoring

of patients with CHF. The veterinary profession would benefit from having a simple diagnostic test that would help identify patients with heart disease and patients with CHF as well as help distinguish patients with respiratory disease from patients with CHF.

In conclusion, this study suggests that plasma [BNP] is high in dogs with moderate to severe MVD alone and is higher in dogs with severe MVD and CHF. This study also suggests that severe increases in plasma [BNP] may be predictive of premature death due to cardiovascular disease, much like in human medicine.

Footnotes

[a] Agilent Sonos 5500 ultrasound machine, Philips Medical Systems, Best, The Netherlands
[b] Canine BNP-32 radioimmunoassay, Peninsula Laboratories Inc, Belmont, CA
[c] StatView, SAS Institute, Cary, NC
[d] StatXact, Cytel Software Corp, Cambridge, MA
[e] Triage BNP Test, Biosite Diagnostics, San Diego, CA

Acknowledgment

This study was funded by the Center for Companion Animal Health at the University of California at Davis.

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EXHIBIT G

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Cardiovascular Research 51 (2001) 601–607

Cardiovascular Research

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Differential expression of cardiac ANP and BNP in a rabbit model of progressive left ventricular dysfunction

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Received 19 December 2000; accepted 26 March 2001

Abstract

Objective: Activation of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) is considered a hallmark of myocardial remodeling. To determine magnitude and relative proportion of activation during the progression to heart failure, we assessed ANP and BNP gene expression in atrial and left ventricular (LV) tissue in a newly developed model of progressive rapid ventricular pacing-induced heart failure in rabbits. **Methods:** Six animals underwent progressive pacing with incremental rates (330 beats per min (bpm) to 380 bpm over 30 days), resulting in congestive heart failure (CHF). Five animals underwent pacing at 330 bpm for 10 days only (early LV dysfunction, ELVD) and five additional animals served as control group (CTRL). **Results:** ELVD was characterized by decreased mean arterial pressure ($P=0.05$ vs. CTRL) as well as significantly impaired LV function (LV fractional shortening (FS) $P<0.01$ vs. CTRL) and dilatation ($P<0.01$ vs. CTRL). CHF was characterized by further decreased mean arterial pressure ($P<0.01$ vs. ELVD), further impaired LV function (FS $P<0.03$ vs. ELVD) and dilatation ($P<0.01$ vs. CTRL). In control animals, significant ANP expression was observed only in atrial tissue ($P<0.02$ vs. BNP) while BNP expression was ubiquitous but marginal (LV $P<0.05$ vs. ANP). In ELVD, activation of ANP (atria and LV $P<0.05$ vs. CTRL) and BNP (atria $P<0.05$ vs. CTRL, LV n.s.) was observed. In CHF, LV-BNP increased further markedly ($P<0.01$ vs. CTRL, $P<0.05$ vs. ELVD) while atrial ANP and BNP expression as well as LV ANP expression remained unchanged (all P=n.s. vs. ELVD). **Conclusion:** The current studies demonstrate differential activation of atrial and LV ANP and BNP under normal conditions and during the progression to heart failure and provide a molecular basis for the superiority of BNP as marker of LV dysfunction and CHF.

Keywords: Gene expression; Heart failure; Natriuretic peptide

1. Introduction

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are genetically distinct hormones of cardiac origin. ANP was originally extracted from atrial tissue [1] and is released from secretory granules in response to atrial stretch [2]. BNP was first extracted from porcine brain [3] but is now also considered a cardiac natriuretic peptide [4,5]. Both, ANP and BNP have established roles in electrolyte and volume-homeostasis [6,7].

In contrast to ANP, which is predominant in atrial myocytes under normal conditions [8,9], controversy persists with respect to the presence and abundance of BNP gene expression in atrial and ventricular myocardium under normal conditions and in early heart failure. Although the opinion that BNP is of primarily ventricular origin was fueled by studies, which have reported strong left ventricular (LV) BNP expression under normal conditions in humans [10] and animals [11], conflicting studies failed to demonstrate significant expression of LV BNP under normal conditions [9,12–14]. The discussion as to the major site of BNP expression is ongoing, particularly since studies which have investigated atrial as

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Time for primary review 30 days.

0008-6363/01/$ – see front matter
PII: S0008-6363(01)00316-9

well as ventricular BNP expression under control conditions and during the progression to congestive heart failure (CHF) are sparse.

Rapid ventricular pacing-induced heart failure is an animal model which allows to reproducibly induce LV dysfunction and dilatation and has traditionally been carried out in large animals, i.e. dogs, pigs, and sheep [15–17]. Recently, rapid ventricular pacing-induced heart failure was also implemented as a small animal model in the rabbit [18–21]. It was our objective to utilize the rabbit model for the first time to define cardiac ANP and BNP gene expression in atrial and ventricular myocardium throughout the progression to CHF. Since we were particularly interested in the temporal pattern of activation, we modified the traditional model of straight pacing and developed a model, which slowly evolves over 30 days from a chronic period of early LV dysfunction (ELVD) to CHF.

The hypothesis of the current investigation was that atrial myocardium is the major site of ANP expression and that BNP is expressed only marginally in atrial and LV myocardium in the absence of LV dysfunction. Further we hypothesized that differential atrial and ventricular recruitment of ANP and BNP occurs during the progression to CHF and that CHF is associated with a particularly strong recruitment of LV BNP. To address this hypothesis, we assessed ANP and BNP gene expression with rabbit-specific cDNAs in atrial and LV myocardium in the absence of LV dysfunction as well as in ELVD and CHF.

2. Methods

2.1. Study protocol

Sixteen male rabbits (chinchilla bastard) were used for the study. Eleven rabbits underwent implantation of a programmable cardiac pacemaker (Medtronic Minix 8340, Minneapolis, MN). Under anesthesia (ketamine 60 mg/kg and xylazine 5 mg/kg i.m.), the right internal jugular vein was dissected and cannulated with a single-lumen central venous catheter (Braun, Germany). The catheter was then advanced into the right ventricle under pressure guidance. A 2 french transvenous screw-in pacemaker lead (Medtronic) was advanced through the catheter into the right ventricular apex and implanted endocardially. The pacemaker was implanted subcutaneously into the right abdominal wall and the pacemaker lead was connected subcutaneously with the pacemaker. To the best of our knowledge, this is the first study in rabbits where rapid ventricular pacing-induced heart failure was induced with a transvenously implanted pacemaker system. All rabbits were allowed to recover for at least 10 days after surgery before the pacemaker was started for the induction of heart failure. Proper pacemaker function was checked intraoperatively, at the time of programming, and subsequently all 10 days. All studies were approved by the governmental animal care committee.

Six rabbits (CHF group) underwent pacing with a stepwise increase of stimulation frequencies over 30 days. During the first 10 days, animals were paced at 330 beats per min (bpm). This protocol results in ELVD, as defined by significant LV systolic dysfunction with cardiac enlargement and decreased perfusion pressure but no clinical signs of heart failure. The pacing rate was then increased to 360 bpm for 10 days and then 380 bpm for another 10 days and ELVD evolved to CHF with further cardiac enlargement and further decreased perfusion pressure together with clinical signs of fluid retention (ascites). A similar stepwise pacing protocol over 38 days in dogs also evolves from ELVD to CHF and results in progressive canine heart failure [13,22–24].

At baseline (control), after being paced at 330 bpm for 10 days (ELVD) and at the end of the protocol (CHF), conscious arterial pressure was measured invasively via the medial ear artery and a 2-D guided M-mode echocardiogram was obtained. At the end of the pacing protocol, rabbits were killed by i.v. euthanasia and tissue was rapidly harvested. Hearts were trimmed on ice, snap frozen in liquid nitrogen and stored at −80°C until further processing.

A second group of five rabbits was paced at 330 bpm for 10 days only and served as tissue donors for the ELVD group and a third group of five normal rabbits served as tissue donors for the control group. Again, invasive hemodynamic measurements and an echocardiogram were obtained to assess cardiac function before animals were euthanized and tissue was rapidly harvested and deep-frozen.

2.2. Analytical methods

For analysis of cardiac natriuretic peptide expression, mRNA was extracted from all atrial and LV samples utilizing a commercially available kit (Fasttrack, Invitrogen). Briefly, tissue was homogenized (Polytron PT 1200) in a detergent-based buffer containing RNAse/Protein Degrader and incubated in a slow-shaking waterbath. DNA was precipitated and sheared and oligo (dT) cellulose was added for adsorption of polyadenylated mRNA. DNA, proteins, cell debris and non-polyadenylated RNA were washed off and mRNA eluted off the oligo (dT) cellulose. The yield of mRNA was determined in a spectrophotometer by absorption of 260 nm UV-light. Approximately 5 μg mRNA per extract were loaded on a 1.2% agarose formaldehyde gel and electrophoresed for 2–3 h at 75 V. The gel was blotted downward overnight (Turbo-Blotter, Schleicher & Schuell) onto a nylon membrane (Maximum Strength Nytran Membrane, Schleicher & Schuell).

As a probe for ANP mRNA, a 393 base-pair partial cDNA specific for rabbit ANP was synthesized. In brief, first-strand cDNA was reverse transcribed from rabbit

atrial mRNA. Then, DNA-amplification was performed by polymerase chain reaction with 20-base oligomers as primers. Amplification temperatures were 95°C for 60 s, 56°C for 120 s and 72°C for 180 s and 35 amplification cycles were performed. CTAACCCAGTGTACAACGCC was used as 5′ primer and GGCTGTTATCTTCGGTAC-CG as 3′ primer, corresponding to nucleotides 157–176 and 531–540 of the published sequence [25]. The resulting DNA was electrophoresed in a 1% agarose gel, resulting in a single sharp band of the predicted length. This band was recovered from the gel and sequenced. The resulting nucleotide sequence was identical to the predicted sequence, thus confirming that the recovered DNA contained a specific cDNA to rabbit ANP mRNA.

As a probe for BNP mRNA, a 750 bp *Eco*R1/*Hin*dIII DNA restriction fragment containing the gene for rabbit BNP (courtesy A.A. Protter, Scios Inc., Sunnyvale, CA, USA) was used.

Fifty ng of both probes were random primed with P32-dCTP (Random Primed DNA Labeling Kit, Boehringer Mannheim Biochemical, Germany) and column-purified. Membranes were prehybridized (QuickHyb Hybridization Solution, Stratagene) for 10 min at 68°C and then hybridized with the labeled probe for 80 min at 68°C. Membranes were then washed stringent (2× SSC/0.1% SDS at 22°C for 5 min, then 0.2× SSC/0.1% SDS at 22°C for 5 min, then 0.2× SSC/0.1% SDS at 55°C for 20 min) and exposed to an X-ray film overnight. To control for loading conditions and mRNA transfer onto the membranes, blots were re-hybridized with a GAPDH probe. The respective autoradiographic bands for ANP, BNP and GAPDH were quantified with a scanning spectrophotometer and ANP and BNP mRNA expressed in arbitrary units as ratio of autoradiographic densities of the respective band and the GAPDH-band.

2.3. Echocardiography

A long and short-axis echocardiogram (HP Sonos 5500, 12 MHz probe) was performed under light sedation (5 mg midazolam i.m.) in a supine position from the left parasternal window. LV end-diastolic (LVEDd) and end-systolic (LVESd) dimensions and diastolic and systolic thickness of the left ventricular anterior wall (AEDth and AESth) and posterior wall (PEDth and PESth) as well as left atrial diameter (LAd) were determined from three repeated 2D guided M-mode tracings using the ASE convention. From those measurements, fractional shortening (FS) was calculated as: FS=(LVEDd−LVESd)/LVEDd.

2.4. Calculation of wall stress

From the blood pressure recordings, three repeated tracings were used for the assessment of peak systolic arterial pressure (SAP), which was used as an estimate of LV systolic pressure. Left ventricular systolic wall stress (LVSWS) was then calculated as [26,27]:

$$\mathrm{LVSWS} = (\mathrm{SAP} \times \mathrm{LVESd}/2)/(\mathrm{PWSth} + \mathrm{AWSth}) \times (1 + (\mathrm{PWSth} + \mathrm{AWSth})/2 \times \mathrm{LVESd}).$$

2.5. Statistical analysis

Results of the quantitative studies were expressed as mean±standard error of the mean. Comparison between the control, ELVD and CHF groups were performed by analysis of variance (ANOVA) followed by Fisher's least significant difference test. Comparison between the atrial and LV tissues as well as between ANP and BNP were performed by paired Student's *t*-test. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Blood pressure, LV function, and LV geometry (Table 1)

In ELVD, systolic (−7% vs. CTRL, $P<0.02$), diastolic (−6% vs. CTRL, P=n.s.) and mean arterial pressure (−7% vs. CTRL, $P=0.05$) were decreased. Significant LV dysfunction was present (FS −26% vs. CTRL, $P<0.01$)

Table 1
Hemodynamic and echocardiographic characteristics of progressive rapid ventricular pacing-induced CHF in rabbits

	CTRL	ELVD	CHF
MAP (mmHg)	75.6±1.9	70.7±1.5[c]	58.5±2.1[a,b]
SAP (mmHg)	87.9±2.0	81.5±1.4[a]	67.0±2.2[a,b]
DAP (mmHg)	69.2±2.0	65.2±1.7	54.3±2.4[a,b]
LAd (mm)	9.7±0.3	11.2±0.7	12.5±0.8[a]
LVEDd (mm)	12.2±0.4	14.7±0.5[a]	15.5±0.6[a]
LVESd (mm)	7.1±0.4	10.2±0.5[a]	11.9±0.7[a,b]
FS (%)	41.9±1.5	31.2±2.3[a]	23.7±2.1[a,b]
AEDth (mm)	2.7±0.1	2.5±0.1	2.3±0.1
AESth (mm)	5.0±0.2	4.1±0.2[a]	3.4±0.2[a]
PEDth (mm)	2.8±0.1	2.7±0.2	2.3±0.1[a,b]
PESth (mm)	4.3±0.1	3.5±0.2[a]	2.8±0.2[a,b]
BW (kg)	3.8±0.1	4.0±0.1	4.2±0.2
Heart (g/kg BW)	1.9±0.1	2.3±0.1[a]	2.3±0.1[a]
LV (g/kg BW)	1.26±0.06	1.25±0.07	1.21±0.04
RV (g/kg BW)	0.40±0.03	0.45±0.03	0.51±0.03
Atria (g/kg BW)	0.25±0.02	0.60±0.02[a]	0.62±0.05[a]

ELVD, early LV dysfunction; CHF, congestive heart failure; MAP, mean arterial pressure; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; LAd, left atrial diameter; LVEDd, LV end-diastolic diameter; LVESd, LV end-systolic diameter; FS, fractional shortening; AESth, anterior wall end-systolic thickness; AEDth, anterior wall end-diastolic thickness; PESth, posterior wall end-systolic thickness; PEDth, posterior wall end-diastolic thickness; BW, body weight. Data expressed as mean±S.E.M.

[a] $P<0.05$ vs. CTRL.

[b] $P<0.05$ vs. ELVD.

[c] $P=0.05$ vs. ELVD, ANOVA.

and LVs were dilated at end-diastole (LVEDd +26% vs. CTRL, $P<0.01$) and end-systole (LVESd +44% vs. CTRL, $P<0.01$). While only a tendency towards wall thinning was observed at end-diastole (AEDth −7% and PEDth −4% vs. CTRL, both P=n.s.), a significant reduction in wall thickness was present at end-systole (AESth −18% and PESth −19% vs. CTRL, both $P<0.01$).

In CHF, systolic (−18% vs. ELVD, $P<0.01$), diastolic (−17% vs. ELVD, $P<0.01$), and mean arterial pressure (−17% vs. ELVD, $P<0.01$) all decreased markedly and significantly further. LV function decreased further as compared to ELVD (FS −24% vs. ELVD, $P<0.03$) and further LV dilatation (LVEDd +5% vs. ELVD, P=n.s., LVESd +17%, $P<0.05$) and wall thinning at end-diastole (AEDth −8% vs. ELVD, P=n.s., and PEDth −15%, $P<0.04$) and end-systole (AESth −17% vs. ELVD, P=n.s., and PESth −20%, $P<0.03$) were observed.

3.2. Body and heart weight (Table 1)

Total body weight increased progressively but not statistically significantly in ELVD (+5% vs. CTRL, P=n.s.) and CHF (+5% vs. ELVD, P=n.s.). Total heart weight was increased in ELVD (+21% vs. CTRL, $P<0.03$) and did not increase further in CHF. Increased heart weight was due to increased atrial weight (+140% in ELVD and +148% in CHF, both $P<0.01$ vs. CTRL) and a tendency towards increased RV weight (both P=n.s. vs. CTRL). LV weight remained unchanged in ELVD (−1% vs. CTRL, P=n.s.) and CHF (−3% vs. ELVD, P=n.s.).

3.3. Atrial ANP and BNP gene expression (Fig. 1)

A positive signal for ANP and BNP mRNA was detected in all atrial samples from control, ELVD and CHF animals. Under control conditions, atrial ANP mRNA expression significantly exceeded the faint BNP expression (+300% vs. BNP, $P<0.02$). In ELVD, significant increases in both, ANP (+160% vs. CTRL, $P<0.05$) and BNP expression (+1354% vs. CTRL, $P<0.01$) were observed with a greater relative increase of BNP expression ($P<0.01$ vs. ANP). No further increases were observed for ANP or BNP expression when ELVD progressed to CHF. But inversely as in control animals, BNP expression now exceeded ANP expression significantly (+75% vs. ANP, $P<0.03$).

3.4. LV ANP and BNP gene expression (Figs. 2–4)

While LV ANP mRNA expression was absent in most control animals, faint signals for BNP mRNA were present and LV BNP expression exceeded ANP expression significantly ($P<0.05$). In ELVD, the onset of significant LV ANP expression as well as increased LV BNP expression (+542% vs. CTRL, P=n.s.) were observed. While LV

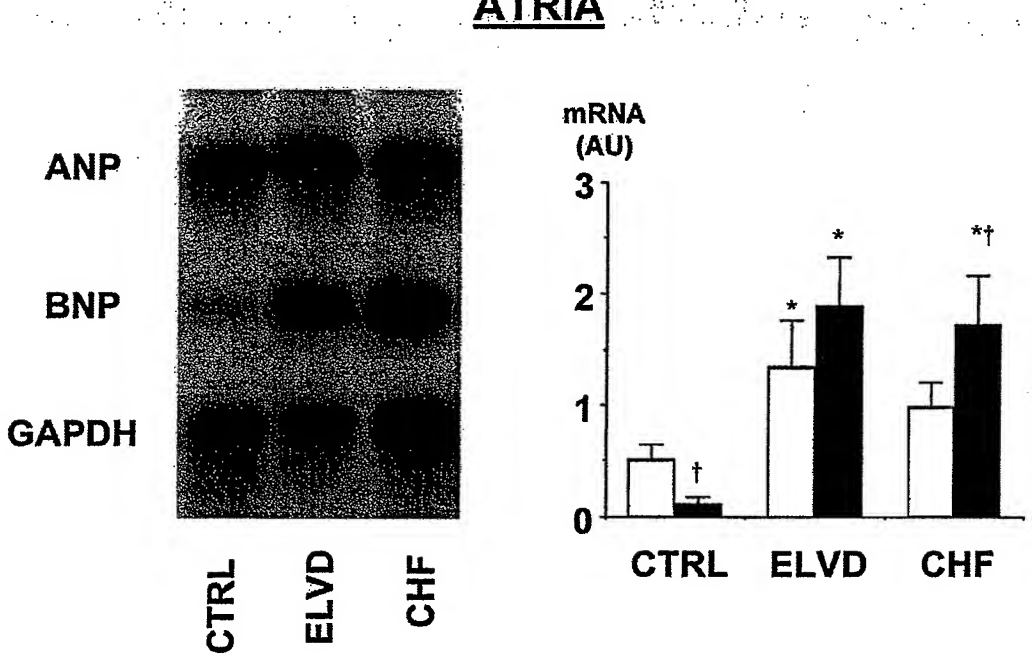

Fig. 1. Left, representative Northern blot for atrial ANP and BNP gene expression. CTRL, control; ELVD, early LV dysfunction; CHF, heart failure. Right, quantitative analysis of atrial ANP (open bars) and BNP (gray bars) gene expression in CTRL (n=5), ELVD (n=5), and CHF (n=6). Data expressed as mean±standard error. *$P<0.05$ vs. CTRL, †$P<0.05$ vs. ANP.

natriuretic peptide in the absence of ventricular dysfunction while BNP is only marginally expressed. In contrast, strong global cardiac natriuretic peptide activation is present during the progression to heart failure with a predominance of BNP in ELVD and CHF.

With respect to activation of BNP, our current findings confirm studies which failed to demonstrate significant expression of LV BNP under normal conditions [9,12] and challenge studies which have reported strong LV BNP expression in normal human [10] and animal [11] myocardium. With respect to the controversy whether BNP expression occurs predominantly in ventricular myocardium, our findings confirm studies that have demonstrated strong activation of LV BNP in severe CHF [9,14] and extend these studies as they demonstrate marked activation of LV BNP already in ELVD. Further they demonstrate additional activation of atrial BNP in ELVD and CHF and that atrial BNP expression in ELVD even exceeds LV expression.

As compared to ANP, activation of LV BNP during the progression of heart failure was observed to be stronger, both in ELVD and CHF. And while LV ANP does not increase further in CHF as compared to ELVD, activation of LV BNP increases further significantly. When related to LV systolic wall stress, it therefore becomes evident that LV BNP expression tracks wall stress closer than ANP expression and does so almost proportionally (Fig. 3). This close association suggests LV BNP expression as a superior marker of LV overload in progressive CHF and provides a molecular basis for the superiority of plasma BNP as biochemical marker of LV dysfunction which is currently evolving in a number of human studies [28–31].

Our finding that full activation of natriuretic peptide gene expression in this model occurs earlier in atrial as compared to LV tissue, namely in ELVD, points to a greater sensitivity of the atrial myocyte to stimulate ANP and BNP gene expression as compared to the ventricular myocyte. This effect may be related to a mechanic mechanism such as the greater atrial distensibility. It may, however, also indicate a greater dependency of ventricular myocytes upon further stimulation such as by local or circulating neurohormones, e.g. ANG II or ET-1. Since ANG II [32] and ET-1 [33] have been shown to induce transcription of early genes and stimulate myocyte growth or even directly stimulate BNP transcription, and since activation of both, ANG II and ET-1, has been demonstrated in clinical [34] and experimental CHF [24,35], these factors might provide additional stimuli in addition to LV systolic wall stress for full expression of natriuretic peptides in LV tissue in CHF.

Although the current study is the first to address cardiac natriuretic peptide gene expression in a small animal model of rapid ventricular pacing-induced heart failure and does so in a newly implemented model of progressive heart failure, cardiac ANP and BNP expression have also been studied earlier in large animal models of pacing-induced heart failure, albeit with conflicting results. When LV ANP expression was first studied in the dog model of traditional straight rapid ventricular pacing, either no induction [8] or only a statistically insignificant trend [17] towards induction of LV ANP gene expression was observed. Similar results were obtained in a very recent study where only weak increases in LV ANP expression were observed after straight pacing in pigs [14]. In contrast, when LV ANP and BNP expression were assessed in a recently developed large animal model of progressive pacing in dogs, strong LV ANP [36] and BNP [13] expression were observed in CHF. However, in contrast to the current study, neither LV ANP nor LV BNP expression was activated in dogs with ELVD [13,36]. The disparity between the current early onset of LV ANP and BNP gene expression in the rabbit model and the late onset in the dog model is remarkable and may indicate that activation of cardiac natriuretic peptide expression in heart failure differs between species. It may, alternatively, also relate to differences in stimulation protocols (with higher pacing rates in the rabbit) or hemodynamic differences. Nevertheless, this observation deserves further attention, particularly in human heart failure. Here, the relative extent of LV ANP and BNP expression in ELVD and CHF has not been established yet and remains to be assessed in future clinical studies.

In summary, the current studies provide insight into the temporal activation of cardiac ANP and BNP expression under normal conditions and in evolving CHF. They demonstrate that ANP is the predominant cardiac natriuretic peptide in the absence of ventricular dysfunction while BNP is only marginally expressed. They challenge the notion that BNP expression occurs predominantly in ventricular myocardium during the progression of heart failure and demonstrate strong atrial activation of BNP in ELVD and CHF. They further demonstrate that while atrial ANP and BNP expression reach an early maximum in ELVD, CHF is characterized by a further increase in cardiac natriuretic peptide activation, predominantly BNP. And lastly, they provide a molecular basis for the superiority of BNP as marker of LV dysfunction based upon the close association of BNP expression with LV systolic wall stress throughout the progression of heart failure.

Acknowledgements

Presented in part at 'Heart Failure 2000', June 29–July 1, 2000, Venice, Italy and published in abstract form in the *European Journal of Cardiac Failure* 2000;2(2):43. Supported by the Deutsche Forschungsgemeinschaft (Lu 562/3-1,2) and by an institutional grant of the Universität Regensburg (ReForM). The authors acknowledge outstanding technical assistance by A. Schiessl.

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